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Characterization of Sourdough Yeasts and Their Application to Low-Alcohol Brewing

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Consumer interest in diverse and innovative beers, as well as the market demand for low-alcohol beers has encouraged more studies into nonconventional yeasts. This has, in turn, led to increased efforts to isolate new yeast strains from different sources. To ensure safety of consumption, potentially applicable sources for new yeasts are fermented foods, such as sourdough.

This thesis was carried out at VTT Technical Research Centre of Finland Ltd, in the Research Area Industrial Biotechnology and Food Solutions. The aim of the thesis was to study sourdough yeasts and assess their potential suitability for low-alcohol brewing. The yeasts used in the study were isolated from diverse *de novo* sourdough cultures produced specifically for this study. Reference yeast strains were obtained from the VTT Culture Collection. Isolated yeasts were later added to the VTT Culture Collection.

Characteristics of different sourdough yeast isolates were determined for suitability in brewing. Fermentation trials were conducted and alcohol production, pH, flavour and carbonyl compounds were measured. Stress factors such as temperature, ethanol and osmotic tolerance were evaluated for each yeast.

Screening of 10 different species from sourdough yeasts indicated their respective suitabilities for brewing. All tested strains produced low levels of alcohol due to an inability to ferment the wort sugar maltose. The results show that most of the non-conventional yeasts are capable of aldehyde reduction and could be used to remove the raw 'worty' flavour notes typical of low-alcohol beers. Production of aromatic volatiles were limited, but a strain of *Kazachstania servazzi,* isolated from rye malt sourdough, showed some potential in this regard, producing high levels of the floral aroma compound phenylethyl acetate. Results suggest that isolation of yeasts from *de novo* sourdough cultures is a feasible strategy for obtaining phenotypically diverse strains for low-alcohol brewing applications.

Keywords	sourdough, yeast, fermentation, low-alcohol beer, bio-prospecting, flavour

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Kuluttajat ovat yhä kiinnostuneempia oluiden ominaisuuksista, ja monipuolisten ja innovatiivisten oluiden kysyntä on lisääntynyt markkinoilla, lisäksi tämä on kannustanut tutkimusten lisääntymistä vaihtoehtoisiin hiivoihin. Tämä on puolestaan johtanut tutkijoiden lisääntyneeseen kiinnostukseen eristää uusia hiivakantoja eri lähteistä. Kulutuksen turvallisuuden takaamiseksi soveltuvat lähteet uusille hiivoille ovat käymisteitse tuotetut elintarvikkeet, kuten hapanleipäjuuri.

Tämä insinöörityö suoritettiin Teknologian tutkimuskeskus VTT:llä, teollisen bioteknologian ja elintarvikeratkaisujen tutkimusosastolla. Insinöörityön tavoitteena oli hapanleipähiivojen tutkiminen ja niiden potentiaalinen soveltaminen vähäalkoholisen oluen valmistukseen. Työssä käytetyt hiivat eristettiin erilaisista hapanleipäviljelmistä, jotka oli tuotettu erityisesti tätä tutkimusta varten. Viitteinä käytetyt hiivat olivat VTT:n kantakokoelmasta. Hapanleipäjuuresta eristetyt hiivat lisättiin myöhemmin VTT:n kantakokoelmaan.

Työssä tutkittiin hapanleipähiivojen ominaisuuksien soveltuvuutta oluen valmistuksessa. Fermentointikokeita suoritettiin ja alkoholin tuotanto, pH, aromi sekä karbonyyliyhdisteet analysoitiin. Hiivakantojen stressitoleranssi tutkittiin lämpötilan, etanolin ja osmoottisen sietokyvyn suhteen.

Kymmenen eri hapanleipähiivalajin seulonta osoitti niiden soveltuvuuden oluen valmistukseen. Kaikki tutkitut hiivat tuottivat matalia alkoholipitoisuuksia, johtuen kyvyttömyydestä fermentoida vierteen sisältävää maltoosia. Tulokset osoittivat, että suurimmalla osalla vaihtoehtoisista hiivoista on kyky pelkistää aldehydiyhdisteitä, eli niitä voitaisiin käyttää ”vierremaisen” maun poistamiseksi. Vierremainen maku on tyypillinen vähäalkoholisissa oluissa. Aromaattisten haihtuvien yhdisteiden tuotanto oli matala, mutta rukiin mallashapanleipäjuuresta eristetty *Kazachstania servazzi* -hiiva osoitti potentiaalia, sillä se tuotti runsaasti kukille tuoksuvaa fenyylietyyliasetaattia. Tulokset viittaavat fenotyyppisesti monipuolisten hiivakantojen mahdollisesta löytämisestä vähäalkoholisen oluen valmistukseen hapanleipäjuuresta eristämällä.

Avainsanat	hapanleipä, hiiva, fermentointi, vähäalkoholinen olut, seulonta, aromi

Contents

List of Abbreviations

NABLAB	Non-alcoholic and low-alcohol beer
YPD	Yeast extract peptone dextrose
DNA	Deoxyribonucleic acid
OD	Optical density
EDTA	Ethylenediaminetetraacetic acid
POF	Phenolic off-flavour
ABV	Alcohol by volume

1 Introduction

Yeast plays an integral part in the brewing process and can have a considerable impact on characteristics such as taste and aroma of the beer. Therefore, the selection of yeast is important for the beer being produced. Despite this, only a limited number of yeast species are commonly used. The most widely used yeasts in brewing are from the *Saccharomyces* genus, such as *S. cerevisiae* and *S. pastorianus*. Alternative yeasts, especially used in the production of craft beer, include *Brettanomyces/Dekkera, Torulaspora* and *Pichia* (Basso et al. 2016; Gschaedler, 2017; Hittinger et al. 2018).

The rising popularity in craft beer and consumer interest in product diversity and new properties, such as interesting aroma and flavour profiles, have encouraged more studies into non-conventional yeasts (Basso et al. 2016; Callejo et al. 2017; Cubillos et al. 2019; Gibson et al. 2017). There are several potential sources for these unconventional yeasts, these include kombucha (Bellut et al. 2018), sourdough (Catallo et al. 2020; Marongiu et al. 2015; Mascia et al. 2015; Ripari et al. 2018; Rossi et al. 2018) and cachaca (Araujo et al. 2018; Figueiredo et al. 2017).

Interest in non-alcoholic and low-alcohol beer (NABLAB) has also been on the rise due to lifestyle trends, new policies and demographics. NABLAB can be produced by dealcoholization of normal strength beers with thermal or membrane methods to remove ethanol, or biological methods like stopped fermentation, or special yeasts to limit ethanol production. The biological method involves usage of yeasts that naturally produce lower levels of ethanol due to their inability to ferment all sugars in the wort (Bellut et al. 2018, 2019; Branyik et al. 2012; Liguori et al. 2015). Yeasts producing low levels of alcohol can be found in fermented foods and beverages such as the previously mentioned products kombucha and sourdough. Isolates from these, or other fermented foods, are ideal due to their presumed safety for consumption.

Previous studies on sourdough yeasts in beer production have mainly focused on *Saccharomyces* strains (Marongiu et al 2015; Mascia et al. 2015). Exploring novel yeasts increase the possibility of finding potentially unique properties that can be utilized in beer

production. Due to their high level of biodiversity, sourdoughs are a promising source for novel yeasts (De Vuyst et al. 2005, 2016; Hammes et al. 2005).

In this study, ten yeasts isolated from diverse *de novo* sourdough cultures were studied and tested for their potential suitability for low-alcohol beer production. Stress factors such as temperature, ethanol and osmotic tolerance were determined for each yeast. The production of phenolic off-flavours was also measured. Fermentation trials were conducted and results evaluated. A low-alcohol beer was produced at 10 L-scale and bottled.

2 Theoretical Background

2.1 Low-alcohol beer

2.1.1 Beer and health

The negative aspects of excessive alcohol consumption are commonly known, as are the problems that come with it, such as increase in violent crime, traffic accidents, public disorder and damage to health. After consumption, the ethanol in the alcoholic beverage is absorbed by diffusion and led into the bloodstream. Ethanol is mainly metabolized in the liver, where it is oxidized to acetaldehyde. Acetaldehyde is highly toxic (Branyik et al. 2012). Common medical problems due to excessive alcohol intake is obesity, cirrhosis, pancreatitis and fetal alcohol syndrome among other birth defects if consumed during pregnancy. Alcohol has negative effects on the nervous system and leads to brain stem degeneration and changes to the brain. Other harmful effects include muscle degeneration, dementia and higher risk of cancer (Bamforth 2002; Branyik et al. 2012; Liguori et al. 2015; Mangindaan et al. 2018).

Despite this, there is strong evidence that moderate alcohol intake has a health benefit in the long-term, even more so than abstinence. Moderate beer or wine consumption has shown a favourable impact on the body, such as reducing the risk of coronary diseases, heart attacks, diabetes and ulcers (Bamforth 2002; Branyik et al. 2012). Moderate alcohol consumption has been linked to better cognitive function in old age and reducing the risk of macular degeneration due to age (Bamforth 2002).

Low-alcohol beer has been shown to be a good source for B vitamins (thiamine, riboflavin, niacin, B6, biotin, folate and B12), minerals (Ca, P, Mg, Fe, Zn, K, Na and Se), soluble fibres and antioxidants. Among these antioxidants and free-radical scavengers are benzoic acid, cinnamic acid, coumarin, catechins, phenols, di-, tri-, and oligomeric proanthocyanidins and flavonoids. These compounds may play a role in preventing cancer, tumors, diabetes, aging and neurological diseases (Bamforth 2002; Liguori et al. 2015; Mangindaan et al. 2018).

Low-alcohol beer has been rising in popularity due to new lifestyle trends and may encourage moderate alcohol consumption. Consumers are getting more health conscious, which can be seen in the beer market (Bellut and Arendt 2019).

2.1.2 Low-alcohol beer market

Consumer interest in non-alcoholic and low-alcohol beer (NABLAB) has been on the rise due to changes in lifestyle trends, new policies and demographics (Bellut and Arendt 2019). The market increase for NABLAB is estimated to continue and is encouraging the development of new products. Studies suggest NABLAB has potential for new sensory properties that would have a place in the beer market (Gibson et al. 2017).

During the years of 2011 and 2016, the world-wide NABLAB market grew 20% in total volume. According to forecasts, the market will grow by another 24% until 2021. Out of the total NABLAB volume growth, non-alcoholic (NAB) grew by 21%. The Middle East, Africa and Western Europe have the biggest NAB markets both in terms of volume and value (Bellut and Arendt 2019).

Western Europe has the largest NABLAB market, with Germany in particular. In 2016, Germany accounted for 41% of total volume in the NABLAB market in the Western European region, in turn accounting for 14% of the world-wide market (Figure 1). This makes Germany the biggest NABLAB market in the world. Germany is followed by Spain with 38% of the Western European NABLAB market share in volume (Bellut and Arendt 2019).

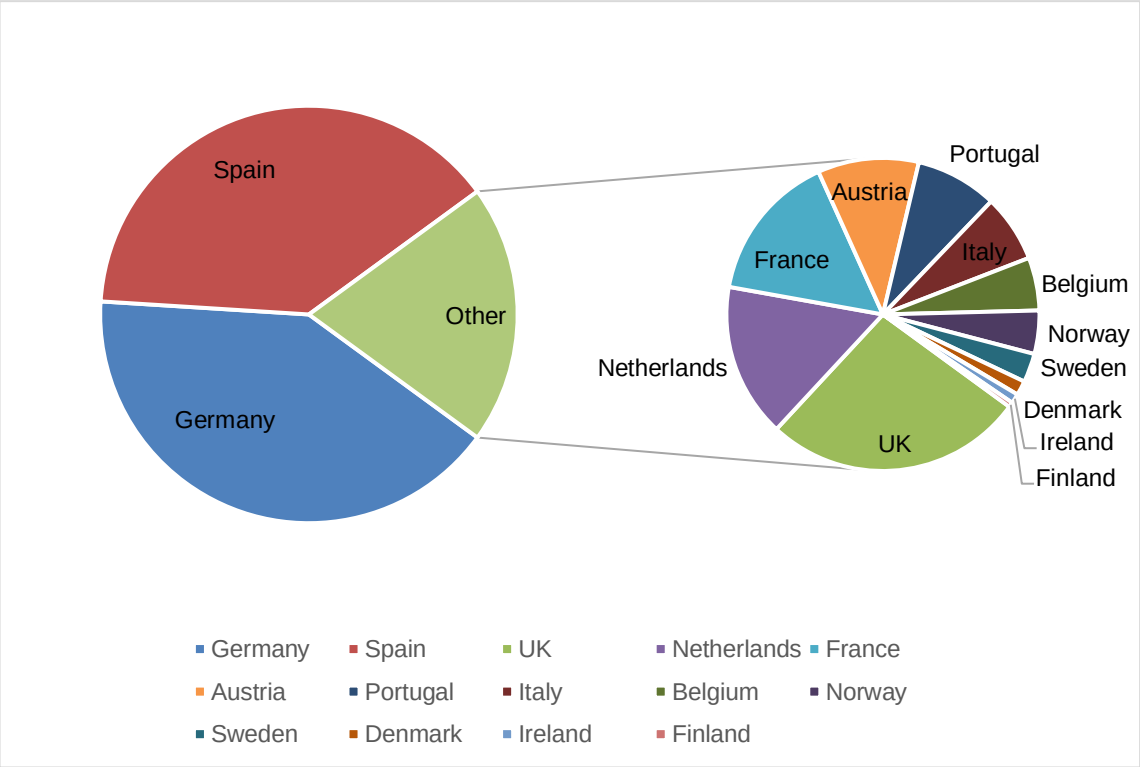

Figure 1. Western European NABLAB market share in volume for individual countries in 2016 (Bellut and Arendt 2019).

One of the reasons for the change in the NABLAB market can be credited to the rising popularity of NAB in the Middle East and North Africa. Alcohol consumption is forbidden in Muslim countries, but in recent times it has become permissible to drink non-alcoholic beverages. NAB allows Muslims to imitate a more Western lifestyle while still following their religious beliefs and rules (Bellut and Arendt 2019).

Another reason for the change is developments in Western consumer attitudes regarding NABLAB. Taste preferences play a key role in beverage consumption. NABLAB have long been associated with inferior taste and aroma compared to more traditional beers (Bellut and Arendt 2019). De-alcoholization of normal strength beers result in the loss of volatile compounds and therefore loss of flavour and aroma (Bellut and Arendt 2019; Branyik et al. 2012; Liguori et al. 2015; Mangindaan et al. 2018). The change in taste can be demonstrated with consumer ratings, where low-alcohol beers are getting lower scores compared to beers with higher alcohol content. The contrast in the ratings can also be due to the association of the consumer, rather than the actual taste of the product. This has been demonstrated in various consumer studies relating to NABLAB. The

emergence and popularity of craft beers has boosted consumer interest in more innovative and diverse beers. This has, in turn, made it feasible for small brewers to explore different aspects of the beer such as alcohol content and has encouraged usage of non-conventional yeasts in brewing. Craft beer is associated with higher quality and diversity in flavour and aroma compared to mass-produced beer. Product improvement and innovation is therefore the right approach to achieve change in the beer market. Additionally, consumers are becoming increasingly health conscious and are aware of the negative effects of excessive alcohol intake. Emerging research into the health benefits of beer and low-alcohol beer are also playing a role in the change (Bellut and Arendt 2019).

2.1.3 De-alcoholization methods

The production methods for non-alcoholic and low-alcohol beers can be divided into two groups, physical and biological processes. The physical process of de-alcoholization involves removing alcohol from regular beer by thermal methods or membrane-based processes. Vacuum evaporation and vacuum distillation are common thermal de-alcoholization methods. Vacuum evaporation, as the name suggest, uses varying methods to evaporate and separate the ethanol from the beer. Two different variants of these are centrifugal and falling film evaporators. Vacuum distillation involves preheating of the beer, degassing and recovery and redirection of the aroma components to the de-alcoholized beer (Branyik et al. 2012). The significant negative impact of thermal methods is how much the final product differs from the regular beer. Many of the volatile compounds are damaged by the heat and the flavour and taste of the beer will differ greatly. Additionally, thermal methods require a large amount of energy for the processes (Mangindaan et al. 2018).

Physical de-alcoholization methods

Membrane-based methods include reverse osmosis, dialysis and osmotic distillation. These methods are based on the semipermeablility of the membranes used. Membranes, depending on their type, can separate different molecules and volatiles. Membrane-based methods can remove the alcohol almost completely. Reverse osmosis (RO)

removes ethanol under a mild temperature (<15 °C) and at pressures ranging from 2 to 8 MPa. The advantages of the low temperature are limiting the damage to temperature sensitive compounds and low energy consumption. The membrane used has high permeability for ethanol and water, but low permeability for components such as flavour and aroma (Branyik et al. 2012; Mangindaan et al. 2018).

The dialysis method is based on diffusion. The ethanol is removed with selective diffusion, where the ethanol diffuses through a semipermeable membrane from the beer into water. This happens due to the difference in concentration between the beer and the dialysate (water). The process is usually performed at 1-6 °C and at pressures ranging from 10 to 60 kPa (Branyik et al. 2012; Mangindaan et al. 2018). In osmotic distillation, the beer is flowing along the surface of the membrane while the stripping solution is flowing in a counter current flow on the other side of the membrane. The ethanol in the beer will permeate the microporous hydrophobic membrane into the stripping solution (water). The process is performed at room temperature and at atmospheric pressure (Mangindaan et al. 2018).

Often, the problem with physical de-alcoholization is the loss of flavour and impact on quality. The physical methods also require specialized equipment for the de-alcoholization, and may necessitate considerable capital expenditure on the part of the brewer (Branyik et al. 2012; Liguori et al. 2015; Mangindaan et al. 2018).

Biological methods for limiting alcohol production

The biological methods focus on limiting the production of ethanol in the beer rather than removing it post-production. These methods include limiting the fermentable sugars in the wort, special yeasts and arrested fermentation. Yeasts produce ethanol by fermenting the sugars in the wort, which means limiting the fermentable sugars in the wort limits the ethanol production. This requires more work in the production of the wort. Using arrested fermentation as a method for low ethanol content means removal of the yeast at low attenuation. The problem with these methods is the negative effects on the flavour and the aroma. These include a strong ‘worty’ off-flavour and a lack of fruity aroma (Branyik et al. 2012; Liguori et al. 2015; Mangindaan et al. 2018).

Special yeasts are those that naturally produce lower levels of ethanol due to their inability to ferment all sugars in the wort. Wort consists of many different sugars, out of which maltose is the most prominent. Other sugars included are glucose, sucrose, maltotriose or fructose. These are in a significantly smaller proportion to maltose. The special yeasts are usually capable of fermenting glucose, sucrose and fructose but not maltose, which leads to lower levels of ethanol production. The commonly used brewer's yeast (*Saccharomyces cerevisiae*) is effective at fermenting most of the sugars in the wort, which is not ideal for low-alcohol brewing (Branyik et al. 2012; Liguori et al. 2015; Mangindaan et al. 2018).

These special yeasts are therefore mostly non-*Saccharomyces* yeasts. Non-*Saccharomyces* yeasts are also referred to as non-conventional yeasts. The most popular commercially used special yeast is *Saccharomycodes ludwigii*, which has been utilized for many years in the production of low-alcohol brewing. There is extensive research on the usage of *S. ludwigii* for low-alcohol brewing, but studies on other non-*Saccharomyces* yeasts has been limited until recently (Bellut and Arendt 2019).

2.2 Non-conventional yeasts

Consumer interest in diverse and innovative beer has encouraged more studies into non-conventional yeasts. These yeasts have the potential for producing beers with distinctive and interesting flavour profiles (Bellut and Arendt 2019; Capece et al. 2018; Gibson et al. 2017). Craft beer currently brings diversity to the beer market. Alternative yeasts used in craft beer include species belonging to *Brettanomyces/Dekkera*, *Torulaspora*, *Pichia* and *Lachancea* (Basso et al. 2016; Gschaedler 2017). Studies have also shown the potential for low-alcohol brewing with yeasts such as *Candida spp., Cyberlindnera spp*., *Hanseniaspora* and *Toluraspora delbrueckii* (Bellut et al. 2018, 2019).

The search for potentially suitable yeast species has led to bioprospecting of wild yeasts, as well as yeasts from other food systems such as bread, wine and kombucha (Cubillos et al. 2019; Gibson et al. 2017). Fermented foods and beverages have been discovered to be good potential sources for non-conventional yeasts, such as kombucha (Bellut et

al. 2018), sourdough (Marongiu et al. 2015; Mascia et al. 2015; Ripari et al. 2018; Rossi et al. 2018) and cachaca (Araujo et al. 2018; Figueiredo et al. 2017). Having foods and beverages as a source for the yeasts is an advantage due to their presumed safety for consumption. Wild novel yeasts in contrast must be comprehensively tested to guarantee safety for utilization in consumable products.

2.3 Sourdough ecology

Sourdoughs have a broad biodiversity consisting of several species of yeasts and bacteria. Together, through their microbial interaction the yeast and lactic acid bacteria form a mutual association that plays a key role in the production process of sourdough. These species are naturally present in the flour or are added as a starter culture.

Most bacteria in sourdoughs are from the genus *Lactobacillus*, such as *L. fermentum*, *L. paralimentarius*, *L. sanfranciscensis* and *L. plantarum*. The most common yeasts found in sourdoughs are *Saccharomyces cerevisiae*, *Pichia kudriavzevii*, *Candida humilis*, *Kazachstania exigua*, *Wickerhamomyces anomalus* and *Torulaspora delbrueckii*. In previous studies, among the identified yeasts in Finnish sourdoughs were *Saccharomyces cerevisiae*, *Candida humilis*, *Wickerhamomyces anomalus*, *Kazachstania exigua*, *Starmerella stellata* and *Kazachstania unispora* (De Vuyst et al. 2005, 2014, 2016; Hammes et al. 2005; Mäntynen et al. 1999).

Sourdough is an appealing source of yeasts due to the high diversity and availability. Novel yeasts isolated from sourdough have potential for unique sensory properties. Additionally, sourdough yeasts are safe for consumption and therefore will not need extensive testing like wild yeasts before being determined as safe. Due to the acidic environment in the sourdough, there is also a lower presence of filamentous fungi. Previous studies applying sourdough yeasts to brewing have mainly focused on *Saccharomyces* strains and the use of non-*Saccharomyces* yeasts from sourdough cultures has not been explored (Catallo et al. 2020; Marongiu et al. 2015; Mascia et al. 2015; Ripari et al. 2018; Rossi et al. 2018).

3 Materials and Methods

3.1 Preparation of sourdough cultures

Sourdoughs were prepared in a sterile beaker by mixing 20 mL flour and 20 mL sterile reverse osmosis water. This was carried out in duplicate for each of ten flour types (Table 1). Beakers were covered with foil and incubated at 25°C.

Table 1. Commercial flours used in the making of the sourdough cultures. Ten cultures with duplicates for each were made. Four types of grain were included.

	Grain	**Brand**
1	Whole grain Graham wheat	Myllyn Paras
2	Organic wheat	Pirkka
3	Wheat	Kinnusen Mylly
4	Wheat	Fazer
5	Organic rye	Myllärin
6	Rye	Myllyn Paras
7	Rye malt (kaljamallas)	Tuoppi Laihian Mallas
8	Rye malt	Viking Malt
9	Barley	Myllyn Paras
10	Oat	Juntulan

The sourdough cultures were further fed each day for a week by adding 10 mL flour and 10 mL reverse osmosis water. The ratio of flour to water was modified depending on the consistency of the sourdough.

3.2 Yeast isolation procedure

The isolation was started by diluting 1 mL of each culture with 9 mL sterile water. A ten-fold dilution series was carried out and 50 µL of dilutions 10^{-3} and 10^{-4} were plated on

2% agar YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar) supplemented with triton, chloramphenicol and chlortetracycline (10% triton 2 mL/L, 10 % chloramphenicol 1 mL/L, 0.4% chlortetracycline 25 mL/L). Duplicates were plated for both dilutions for each culture. Plates were incubated at 25°C and colony formation was monitored. Individual colonies were selected and transferred to new plates depending on morphology. Representative isolates were transferred to new plates.

3.3 Sequencing and identification

DNA from the representative isolates was extracted by suspending a fresh colony of the yeast in 50 µL DNA extraction buffer (2 mM NaOH, 0.001% Sarcosine), boiling at 95°C for 10 min and centrifuging (1 min at 5000 rpm). The supernatant containing the genomic DNA crude extract was collected. ITS PCR/RFLP was performed by using primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and digested using the HaeIII restriction enzyme (New England BioLabs, USA). Isolates were sequenced at Microsynth Seqlab (Germany).

3.4 Yeast strains

Thirteen different yeast strains were used in this study (Table 2). Ten of the strains had been isolated from self-made sourdoughs and were selected after sequencing. Strains A15 (A-63015), A62 (A-81062) and *Saccharomycodes ludwigii* (C-181010) from the VTT Culture Collection were used as reference strains. Cultures were maintained on standard 2% agar YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar).

Table 2. Yeast strains used in the study. Ten of the yeasts were isolated from the prepared sourdough cultures. Three yeasts were from the VTT Culture Collection. The yeasts isolated during this study were added to the VTT Culture Collection. Strains highlighted in bold were selected for the second fermentation.

VTT Code	Code	Abbreviation	Strain	Source
VTT C-191028	7b B14	Cf	*Cyberlindnera fabianii*	Rye malt sourdough, this study
VTT C-191029	1a 10-4 B2	Hu	*Hanseniaspora uvarum*	Wheat sourdough, this study
VTT C-191027	7a 10-4 A3	Ks	***Kazachstania servazzi***	Rye malt sourdough, this study
VTT C-191030	6b B15	Km1	*Kluyveromyces marxianus*	Rye sourdough, this study
VTT C-191031	9b A2	Km2	*Kluyveromyces marxianus*	Barley sourdough, this study
VTT C-191032	1a B2	Pf1	*Pichia fermentans*	Wheat sourdough, this study
VTT C-191033	6a A13	Pf2	***Pichia fermentans***	Rye sourdough, this study
VTT C-191034	8b A14	Pk	*Pichia kudriavzevii*	Rye malt sourdough, this study
VTT A-81062	VTT A81062	A62	*Saccharomyces cerevisiae*	VTT Culture Collection
VTT A-63015	VTT A-63015	A15	*Saccharomyces pastorianus*	VTT Culture Collection
VTT C-181010	VTT C-181010	C1010	***Saccharomycodes ludwigii***	VTT Culture Collection
VTT C-191035	5a 10-3 B1	Td1	*Torulaspora delbrueckii*	Rye sourdough, this study
VTT C-191036	8b A13	Td2	*Torulaspora delbrueckii*	Rye malt sourdough, this study

The yeasts in the study were selected based on diversity. The yeasts of the same species were isolated from different sourdough cultures and/or grain types (Table 2.). One of the *Pichia fermentans* yeasts was isolated from a wheat culture, the other from rye. The *Kluyveromyces marxianus* yeasts were isolated from rye and barley cultures. Both

Torulaspora delbrueckii yeasts were isolated from rye cultures, but the other one of them was made with rye malt. The isolated yeasts used in the study were later added to the VTT Culture Collection.

3.5 Yeast stress tolerance

Stress tolerance tests were performed on the strains using spot plates. Colonies of the strains were inoculated into 25 mL standard growth medium (YPD 2%) and incubated for 4 days. OD (600 nm) was measured and corrected to 0.5 with sterile water and a ten-fold dilution series was carried out. 5 µL of each dilution was spotted onto YPD plates.

Temperature tolerance tests were conducted on standard 2% agar YPD plates (1% w/v yeast extract, 2% w/v peptone, 2% w/v glucose and 2% w/v agar) and incubated at 15°C, 25°C and 37°C.

Ethanol tolerance was tested on a standard 2% agar YPD plate supplemented with 10% v/v ethanol and incubated at 25°C. To assess osmotic tolerance, a 4% agar YPD plate supplemented with 20% v/v sorbitol was used and incubated at 25°C.

3.6 Phenolic off-flavour

Colonies were inoculated from YPD plates into 25 mL of standard growth medium (YPD 2%) supplemented with 100 mg/L of ferulic acid. The cultures were incubated for 6 days at 25°C, with shaking (80 rpm). After incubation, absorbance was measured for each sample supernatant at 325 nm wavelength. Positive and negative controls were included in the test (strains A62 as positive and A15 as negative control), as well as a blank measurement (uninoculated YPD 2% medium with ferulic acid). Each strain was tested with two biological replicates.

3.7 Fermentation trials

The first fermentation trial was carried out in duplicates, in 2 L cylindroconical stainless steel fermenting vessels, containing 1.5 L of wort medium. The second fermentation trial was carried out in duplicates in 10 L cylindroconical stainless steel fermenting vessels containing 8 L wort medium. The 12°P wort, for the first fermentation trial, was produced at the VTT Pilot Brewery from pilsner malt. The 8°P wort, for the second fermentation trial, was also produced at the VTT Pilot Brewery from pilsner malt. The yeasts were inoculated at a rate of 10 x 10^6 and 1 x 10^6 viable cells mL^{-1} to the 12°P and 8°P worts, respectively. The fermentations were carried out at 25°C for 162 h for the first fermentation and 143 h for the second. Wort samples were drawn at 22, 42 and 162 h for the first fermentation and at 1, 22, 46, 70, 94, 119 and 143 h for the second. Samples were taken from the vessels aseptically and placed directly on ice.

3.8 Wort and beer analysis

The specific gravity, pH and alcohol level (% v/v) of the samples were determined from the centrifuged and degassed samples using an Anton Paar Density Metre DMA 5000 M with Alcolyzer Beer ME and pH ME modules (Anton Paar GmbH, Austria).

3.8.1 Analysis of flavour compounds

Flavour compounds were measured by headspace gas chromatography with flame ionization detector (HSGC-FID) analysis. 4 mL of each sample was filtered (0.45 µm) and incubated at 60°C for 30 min, after which 1 mL of gas phase was injected into the chromatograph equipped with an FID detector and headspace autosampler (Agilent 7890 Series; Paulo Alto, CA, USA). The analytes were separated on a HP-5 capillary column (50 m × 320 µm × 1.05 µm column; Agilent, USA). Helium was used as the carrier gas (constant flow of 1.4 mL min^{-1}). The program used for the temperatures was 50°C for 3 min, 10°C min^{-1} to 100°C, 5°C min^{-1} to 140°C, 15°C min^{-1} to 260°C and isothermal for 1 min. Identification of the compounds was determined by comparison with authentic standards and standard curves were used for quantification. The internal standard used was 1-Butanol (Krogerus et al. 2016).

3.8.2 Analysis of carbonyl compounds

A headspace sampler (Agilent 7697A) coupled with a gas chromatograph (Agilent 7890B) was used to analyze carbonyl compounds, which were detected using a Micro Electron Capture Detector (HS-GC-ECD). The carbonyl compound standards used were 2-methylbutanal, 2-methylpropanal, 3-methylbutanal, furfural, hexanal, methional, phenylacealdehyde and (*E*)-2-nonenal (Aldrich). A stock solution containing a mixture of the standards in ethanol was prepared at 1000 µg/L for each. The calibration range was 0.5-40 µg/L and the dilutions were prepared in 5% ethanol. The sum of the peaks of the geometrical isomers (E and Z) was used for the calculations. The correlation coefficient (R^2) values were 0.995-0.9999. A solution of the derivatization agent *O*-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine (PFBOA) (Sigma-Aldrich) was prepared at the concentration of 6 g/L. 100 µL of this solution mixed with 5 mL of deionized water was poured into a 20-mL glass vial and sealed with a crimp cap (Agilent). The vials with the sample and standards were placed in the headspace sampler, where the vial was held in the oven at 60°C for 30 min for sample equilibrium, then the sample was injected for 1 min pressurized at 25 psi. The loop temperature was 100°C and the transfer line was held at 110°C. The analytes were separated on a HP-5 capillary column (50 m × 320 µm × 1.05 µm column; J&W Scientific, USA). Helium was used as the carrier gas (flow rate 1.0 mL/min). The front inlet temperature was held at 250°C and injection was in split mode at ratio 10:1. The temperature program used for the oven was 40°C for 2 min, followed by an increase of 10°C/min to 140°C (held for 5 min) and finally an increase of 7°C/min to 250°C (held for 3 min) (Gibson et al. 2018).

3.9 Helm assay

Flocculation of the yeast strains was evaluated by following the procedure of the Helm assay from the 8th edition of ASBC methods of analysis (American Society of Brewing Chemists 1992). Yeast strains were inoculated into 50 mL YP medium with maltose and glucose (2% v/v) and incubated at 25°C for 5 days. 10 mL of the cultures were transferred into centrifuge tubes (control and test sample), centrifuged and supernatant discarded. For the control tube, 9.9 mL of reverse osmosis water and 0.1 mL of 0.5M EDTA was added and then vortexed for 10 seconds. 1 mL of solution, taken just below the meniscus, was added to 9 mL of RO water and absorbance was measured (OD600). For the

test tube, 10 mL of washing solution (0.51 g $CaSO_4$ in 1L of RO water) was added to the tube, vortexed for 10 seconds and centrifuged. The supernatant was discarded and pellet resuspended in 10 mL flocculation solution (0.51 g $CaSO_4$, 6.8 g CH_3COONa, 4.05 g CH_3COOH in 1 L of RO water). The solution was vortexed for 10 seconds, inverted 5 times and left for 6 minutes for the yeast cells to sediment. 1 mL of solution, taken just below the meniscus, was added to 9 mL of RO water and absorbance was measured (OD600). Flocculation was calculated according to the following equation (1):

$$\text{Flocculation (\%)} = (A-B) \times (100/A) \quad (1)$$

A is the OD value of the control

B is the OD value of the test sample

Example:

Flocculation (%) = (1.2495-1.2000) x (100/1.2495)

= 3.96 ≈ 4.0 %

The Helm assay was performed in replicates.

4 Results

4.1 Yeast isolation

Colonies on each plate were counted and the number of viable cells were calculated (Table 3.). Viable cells were not possible to be calculated in Sample 2 cultures due to one replicate containing mold and the other not having any growth. One of the replicate cultures of Sample 5 also contained mold but colonies could be calculated on the other replicate. Both Sample 2 and Sample 5 cultures were made with organic flour, wheat and rye, respectively. Culture 10, made with oat flour, had the lowest number of viable cells, 6.4 x 10^4 mean cell count / ml. No strains isolated from this culture were selected for sequencing. The highest count of viable cells was in culture 7, with 1.9 x 10^9 mean cell count / ml.

Table 3. Calculated number of viable cells in the sample cultures. The yeasts were incubated on YPD plates supplemented with triton, chloramphenicol and chlortetracycline. Viable cells could not be calculated in sample 2 due to mold and lack of growth. Cell numbers are means of two replicates.

Sample	Mean cell count / ml	Flour type
1	4.0×10^7	Whole Grain Graham Wheat
2	mold	Organic wheat
3	6.3×10^6	Wheat
4	1.3×10^6	Wheat
5	2.4×10^8	Organic rye
6	4.4×10^7	Rye
7	1.9×10^9	Rye malt
8	3.5×10^8	Rye malt
9	2.2×10^6	Barley
10	6.4×10^4	Oat

Yeasts isolated from 6 out of the 10 cultures were included in this study. Yeasts used were selected from wheat, rye and barley cultures. No yeasts from cultures 2, 3, 4 or 10 were used due to mold or low cell counts.

4.2 Sequencing and identification

Several different species were identified from the isolated yeast colonies (Appendix 1. Table 12.). The sourdoughs had varied levels of diversity. In two of the sourdough cultures, only one yeast was identified, while in another culture up to five different yeast species were found. No yeasts were sequenced from cultures 2 and 10, which were contaminated with mold.

The genus *Pichia* was found in the cultures with the highest frequency, specifically the strains *P. kudriavzevii* and *P. fermentans*. Out of all 46 separately identified strains 17 were either one of these two previously mentioned strains. The most widespread strain overall was *P. kudriavzevii*, which was found in all grain types (wheat, rye and barley)

but not in all different brands. The yeast was not found in the wheat cultures with the wheat flour used from brands Kinnusen Mylly and Fazer, nor in the organic rye flour from Myllärin.

W. anomalus was dominant in both duplicates of the wheat culture 4 and was the only yeast identified in these. Only the yeast *T. delbrueckii* was identified in the organic rye culture number 5. The most diverse culture, with five different yeasts identified, was the whole grain graham wheat culture.

The yeasts only identified once out of the sequenced yeasts were *H. uvarum*, *K. servazzi* and *W. ciferrii*. The first mentioned was found in the whole grain graham wheat culture, meanwhile the two others were found each in one of the rye malt replicates.

4.3 Yeast stress tolerance

Temperature and stress tolerance were tested with spot plates. Assessing the growth of strains in different conditions is integral in their selection for their intended purposes. Strains growing well at 37°C have a risk of being pathogens, which is why this is important to establish before use. Yeast growing well at 15°C could be used in low-temperature fermentation and could survive in lower temperature conditions while stored in the brewery. Determining the ethanol tolerance of yeast is important in brewing. The advantage of a lower ethanol tolerance is easier handling in the brewery, equipment and surfaces can effectively be disinfected and there is a significantly lower risk of cross contamination. Assessing the osmotic tolerance is significant to determine if the yeast can tolerate stress during fermentation, such as a high level of sugar in wort. If the tolerance is low, the strength of the wort needs to be reduced.

4.3.1 Temperature

The strains reacted differently to the incubation temperatures (Fig. 2). *K. marxianus* and *P. kudriavzevii* preferred 37°C and grew more rapidly than at 25°C. At 37°C, they grew well at all densities but barely had any growth in optical density 0.005 in the control

(25°C). The yeast *H. uvarum* was also heat tolerant. 7 of the 12 strains were, however, unable to grow at 37°C.

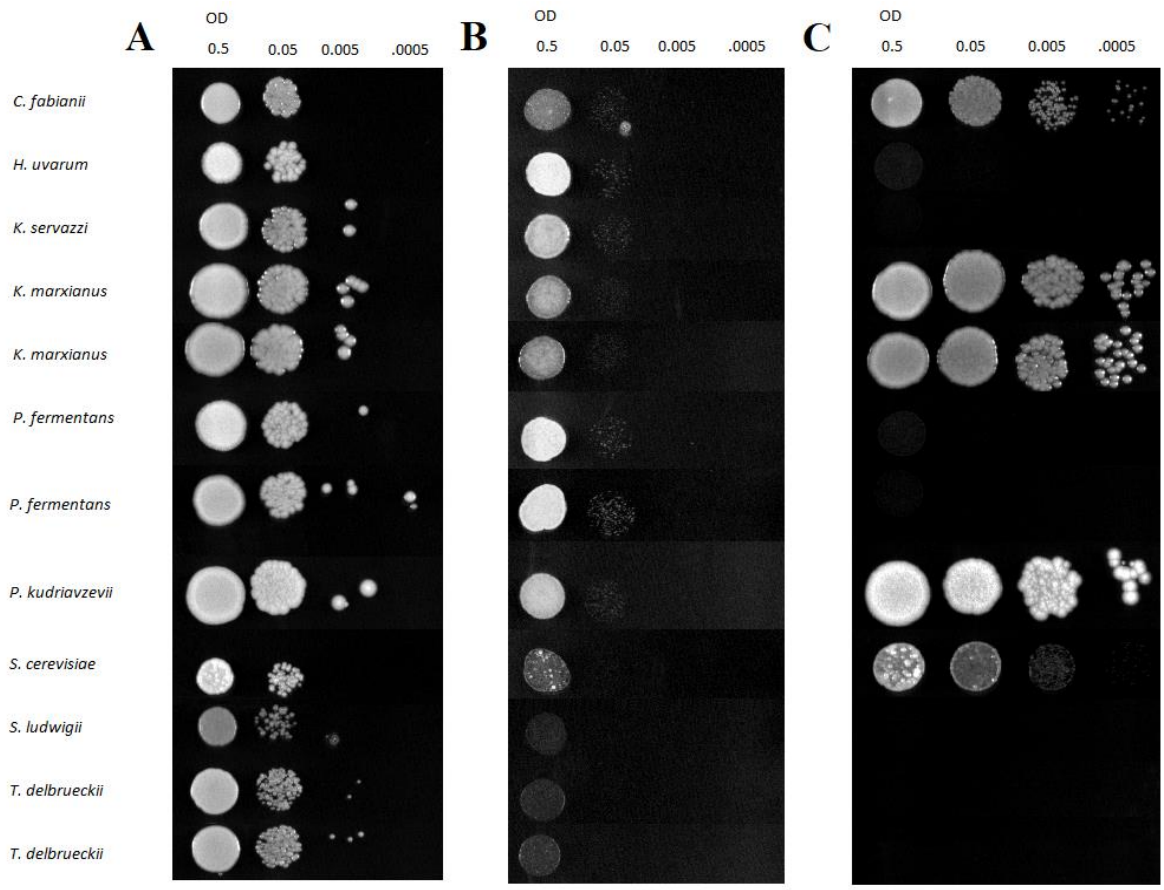

Figure 2. Growth of yeasts on YPD plates incubated at 25°C (A), 15°C (B) and 37°C (C). Plate A and C were incubated for 1 day and plate B was incubated for 2 days, due to the lower temperature. 5 µL of each dilution was spotted on the plate.

Strains selected for the second fermentation, *K. servazzi*, *P. fermentans* and *S. ludwigii*, were all unable to grow at 37°C. All strains grew at 15°C but slower than in the control. All strains grew well at the control temperature 25°C at both 0.5 and 0.05 optical density (Fig. 2.).

4.3.2 Ethanol

The yeast *K. servazzi* had the lowest ethanol tolerance, compared to the other strains that were able to grow at 0.5 optical density without problem (Fig. 3). *P. kudriavzevii* had a higher tolerance of ethanol than the other strains. All strains except *K. servazzi* still had growth at the lower density 0.05 OD.

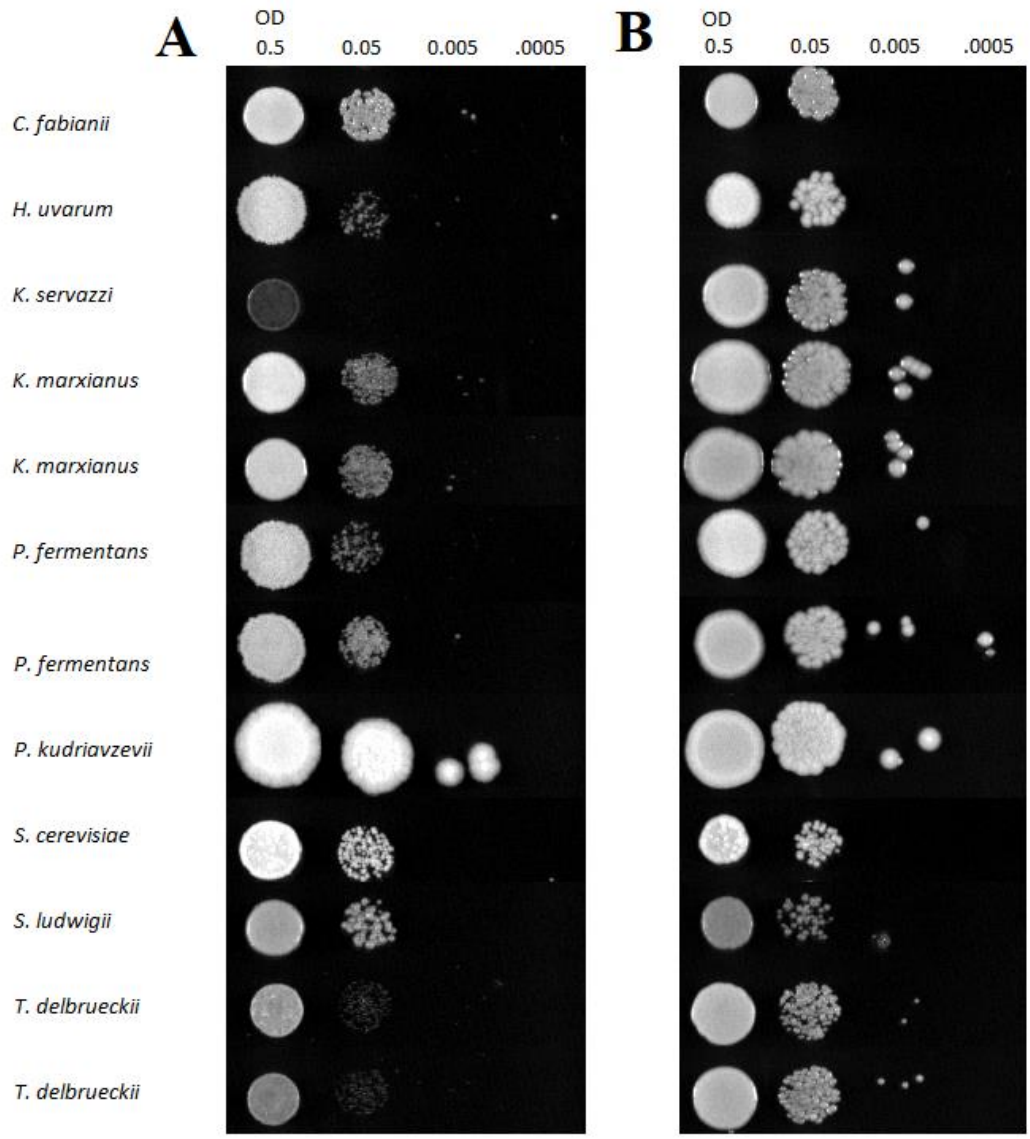

Figure 3. Growth of yeasts on YPD plate containing 10% (v/v) EtOH incubated at 25°C (A) and reference YPD plate incubated at 25°C (B). Both plates were incubated for 1 day. 5 µL of each dilution was spotted on the plate.

The yeasts with least difference in tolerance compared to the reference (B) was *C. fabianii*, *P. kudriavzevii* and *S. cerevisiae* (Fig. 3.).

4.3.3 Osmotic tolerance

All strains showed sensitivity to exposure to 20% (v/v) sorbitol, with *P. kudriavzevii* displaying highest tolerance (Fig. 4.). The *S. ludwigii* strain showed the highest level of sensitivity to osmotic stress, with both *T. delbrueckii* strains displaying similar high sensitivity.

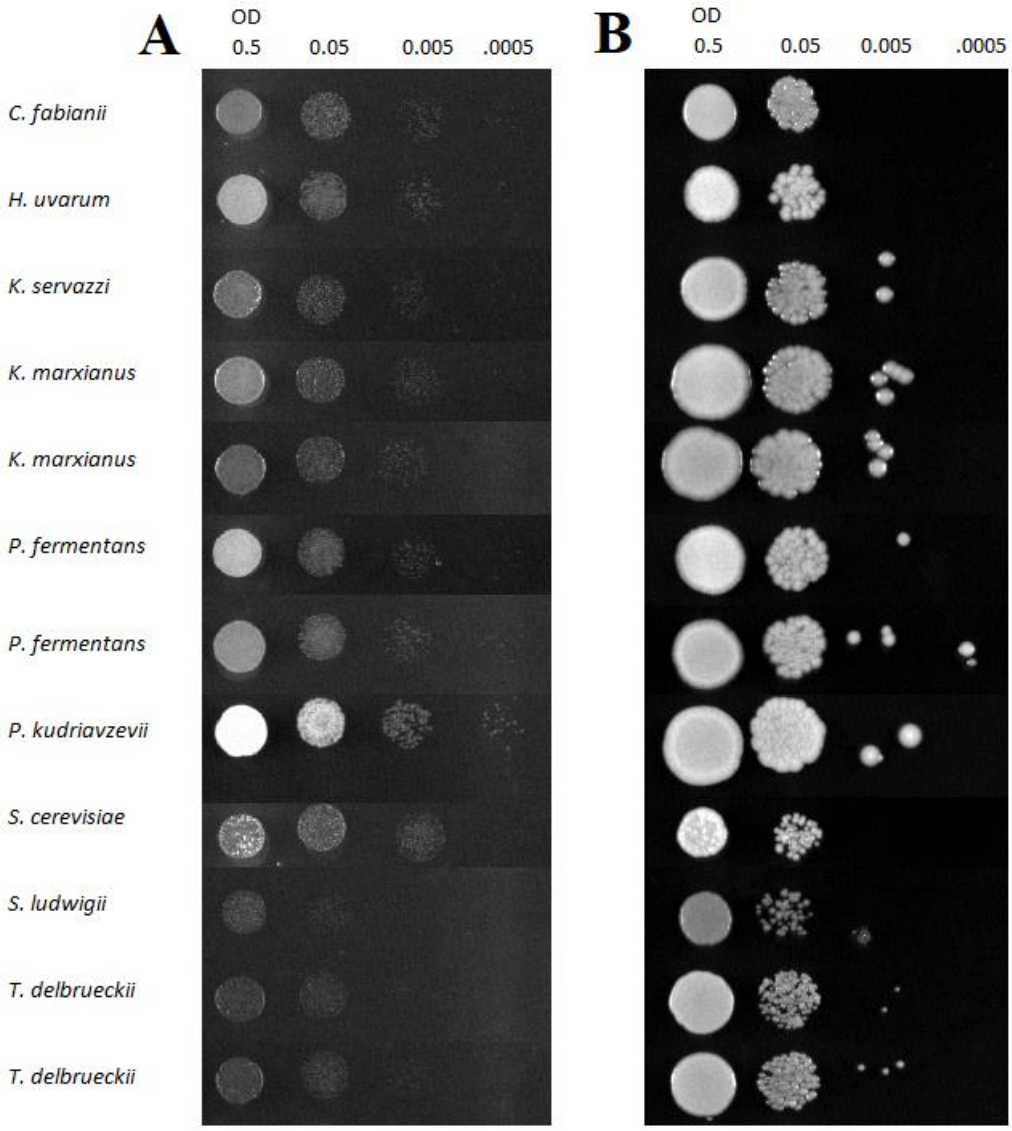

Figure 4. Growth of yeasts on YPD plate containing 20% (v/v) sorbitol incubated at 25°C (A) and reference YPD plate incubated at 25°C (B). Both plates were incubated for 1 day. 5 µL of each dilution was spotted on the plate.

Out of the three strains selected for the second fermentation, *P. fermentans* displayed the highest tolerance for osmotic stress (Fig. 4.).

4.4 Phenolic off-flavour

POF was assessed by measuring the absorbance after 6 days of incubation. By comparing the test strains with the measured references, *H. uvarum* and *P. fermentans* were determined as POF positive (POF^+), while the remaining strains were POF negative (POF^-) (Table 4.). Phenolic off-flavour determines what kind of projects and purposes the yeast strains can be used for.

Table 4. Phenolic off-flavour absorbance results. POF positive results are highlighted in bold. Values are means of two physical replicates with standard error indicating range.

Reference	**Absorbance (325 nm)**	**POF**
A15 (POF^-)	0.54 ± 0.01	-
A62 (POF^+)	**0.49 ± 0.02**	**+**
Samples		
Cyberlindnera fabianii	0.57 ± 0.0	-
Hanseniaspora uvarum	**0.42 ± 0.01**	**+**
Kazachstania servazzi	0.57 ± 0.0	-
Kluyveromyces marxianus	0.56 ± 0.0	-
Kluyveromyces marxianus	0.57 ± 0.01	-
Pichia fermentans	**0.42 ± 0.01**	**+**
Pichia fermentans	**0.41 ± 0.0**	**+**
Pichia kudriavzevii	0.57 ± 0.0	-
Saccharomycodes ludwigii	0.56 ± 0.01	-

Torulaspora delbrueckii	0.59 ± 0.01	-
Torulaspora delbrueckii	0.57 ± 0.0	-

Out of the three strains selected for the second fermentation, *P. fermentans* was POF^+ while *K. servazzi* and *S. ludwigii* were POF^- (Table 4.).

4.5 Fermentation trials

Two fermentation trials were conducted to assess the suitability of the strains for low-alcohol brewing. Among the measurements taken were alcohol, pH, aldehydes and aroma. All yeasts, except the *S. cerevisiae* (A62) control strain, fermented the wort to 0.77%-1.03% alcohol by volume (ABV) after 162 h (Fig. 5). *S. cerevisiae* fermented the wort to 4.79% ABV. Conversion of sugars was therefore low compared to the control yeast. The lowest ABV, 0.77%, was for *P. kudriavzevii*.

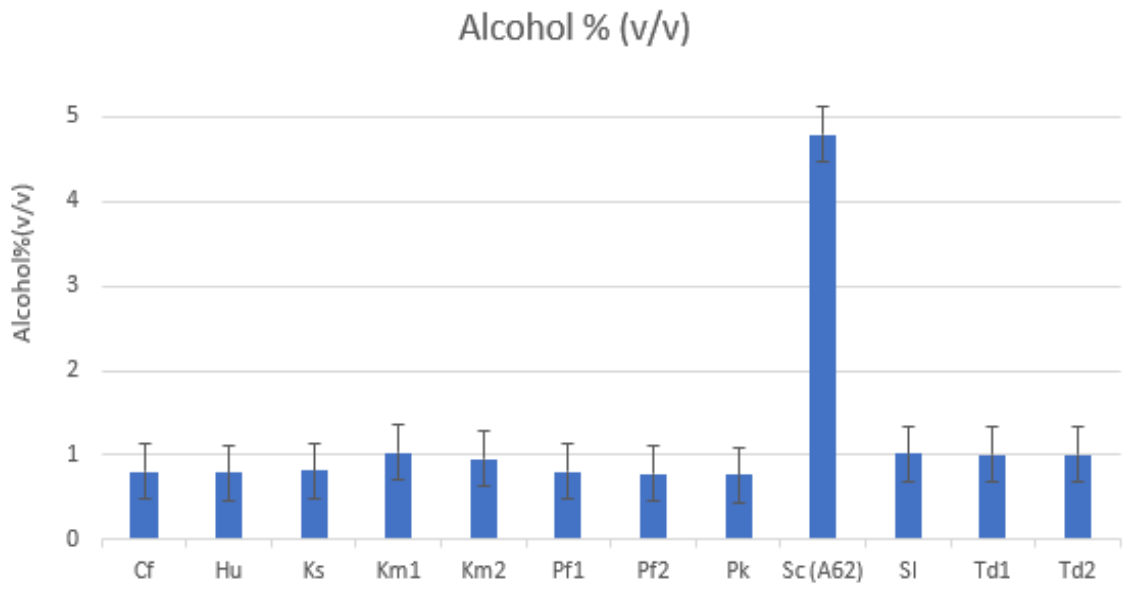

Figure 5. Alcohol content (% alcohol by volume [ABV]) in the green beers fermented on a 2-liter scale from 12 °P wort at 25°C. Samples were taken after 162 h of fermentation. Values are means from two independent fermentations and error bars represent the range. Sc (A62) was the reference strain.

The pH was measured from the green beers, all of which ranged between 4.60 and 4.75 except for the control S. cerevisiae, that had the pH 4.3 (Table 5.). Apparent attenuation was 12.9%-17.3% for all yeasts except *S. cerevisiae*, which had an apparent attenuation of 76.1 %.

Table 5. pH and apparent attenuation in the green beers fermented on a 2-liter-scale from 12 °P wort at 25°C. Samples were taken after 162 h of fermentation. Values are means from two independent fermentations with standard error indicating range.

Samples	**pH**	**Apparent attenuation %**
C. fabianii	4.7 ± 0.0	13.5 ± 0.2
H. uvarum	4.7 ± 0.0	13.0 ± 0.0
K. servazzi	4.7 ± 0.0	13.9 ± 0.1
K. marxianus	4.6 ± 0.0	17.3 ± 0.1
K. marxianus	4.6 ± 0.0	16.4 ± 0.4
P. fermentans	4.7 ± 0.0	13.4 ± 0.1
P. fermentans	4.7 ± 0.0	13.0 ± 0.1
P. kudriavzevii	4.8 ± 0.0	12.9 ± 0.0
S. cerevisiae	4.3 ± 0.1	76.1 ± 0.3
S. ludwigii	4.7 ± 0.0	17.0 ± 0.2
T. delbrueckii	4.7 ± 0.0	17.0 ± 0.1
T. delbrueckii	4.6 ± 0.0	17.0 ± 0.1

Aldehyde production and reduction was measured after 24 h of fermentation, at which point the linear aldehydes acetaldehyde, hexanal and methional were mostly reduced (Table 6.). However, the methional value for all except three strains still exceeded the threshold value. The strains below the threshold were *H. uvarum*, *S. cerevisiae* and one of the *T. delbrueckii* (Td2) strains. Acetaldehyde was measured at 69.8 mg/l for one of the K. marxianus (Km2) strains, which exceeded the 25 mg/l threshold value.

Table 6. Linear aldehyde production in the beers after 24 h during 12 °P wort fermentation at 25°C. Compared to the values in the wort. Values exceeding the threshold value are highlighted in bold. Values are means from technical replicates with standard error indicating range.

Aldehydes (mg/l)	**Acetalde-hyde**	**Hexanal**	**Methional**
C. fabianii	2.7 ± 0.3	<1ppb	**7.4 ± 4.6**
H. uvarum	1.8 ± 0.1	<1ppb	3.9 ± 0.8
K. servazzi	1.8 ± 0.2	<1ppb	**8.1 ± 0.5**
K. marxianus	0.6 ± 0.1	<1ppb	**6.4 ± 1.4**
K. marxianus	**69.8 ± 8.9**	<1ppb	**12.4 ± 2.8**
P. fermentans	1.5 ± 0.2	<1ppb	**6.2 ± 0.6**
P. fermentans	1.7 ± 0.1	<1ppb	**6.5 ± 5.0**
P. kudriavzevii	3.1 ± 0.3	<1ppb	**10.0 ± 0.5**
S. cerevisiae	13.3 ± 1.1	<1ppb	2.1 **±** 0.3
S. ludwigii	2.0 ± 0.2	<1ppb	**11.7 ± 1.0**
T. delbrueckii	3.4 ± 0.5	<1ppb	**4.4 ± 1.7**
T. delbrueckii	2.3 ± 0.2	<1ppb	1.8 **±** 0.1
Wort		1.6 ± 0.1	**176.6 ± 11.8**
Threshold	25 mg/l	88 ppb	4.2 ppb

Production of branched-chain aldehydes was more varied, with values decreasing for most strains except *K. marxianus* and *S. ludwigii* (Table 7.). 2-methylbutanal and 2-methylpropanal increased from 14.7 mg/l and 26.9 mg/l, respectively, to 24.8 mg/l and 143.3 mg/l in the wort with *K. marxianus*. 3-methylbutanal and phenylacetaldehyde increased from 38.6 mg/l and 34.2 mg/l, respectively, to 66.1 mg/l and 248.3 mg/l in the wort with *S. ludwigii*.

Table 7. Branched-chain aldehyde production in the beers after 24 h during 12 °P wort fermentation at 25°C. Compared to the values in the wort. Values exceeding the threshold value are highlighted in bold. Values are means from technical replicates with standard error indicating range.

Aldehydes (mg/l)	**Benzaldehyde**	**Furfural**	**Phenylacetaldehyde**	**2-Methylbutanal**	**2-Methylpropanal**	**3-Methylbutanal**
C. fabianii	0.3 ± 0.0	1.7 ± 0.1	2.8 ± 0.3	0,8 ± 0.1	5.3 ± 0.4	1.0 ± 0.1
H. uvarum	0.3 ± 0.0	0.9 ± 0.1	9.7 ± 2.7	1.7 ± 0.3	10.7 ± 1.1	4.3 ± 0.8
K. servazzi	0.3 ± 0.0	0	4.6 ± 1.6	3.7 ± 0.8	5.8 ± 0.9	15.7 ± 3.5
K. marxianus	0.3 ± 0.0	4.4 ± 0.0	3.3 ± 1.0	23.1 ± 4.0	**143.4 ± 22.3**	6.4 ± 1.2
K. marxianus	0.3 ± 0.0	5.9 ± 0.6	4.3 ± 0.9	26.5 ± 4.2	**143.2 ± 17.0**	9.7 ± 1.6
P. fermentans	0.3 ± 0.0	0.6 ± 0.1	14.8 ± 2.7	**3.1 ± 0.3**	**23.4 ± 2.5**	**10.9 ± 1.4**
P. fermentans	0.3 ± 0.0	0.6 ± 0.1	17.1 ± 3.4	**2.8 ± 0.3**	**21.9** ± 1.3	**10.6 ± 1.2**
P. kudriavzevii	0.3 ± 0.0	1.2 ± 0.0	22.4 ± 5.1	**9.6 ± 0.8**	**20.2** ± **0.7**	**34.8 ± 3.8**
S. cerevisiae	0.3 ± 0.0	0.5 ± 0.2	8.4 ± 0.9	**5.5 ± 0.2**	**6.0** ± **0.4**	**13.8 ± 0.6**
S. ludwigii	0.4 ± 0.0	0.3 ± 0.1	**248.3 ± 54.5**	**10.5 ± 10.4**	**34.1 ± 4.3**	**66.1 ± 12.3**
T. delbrueckii	0.3 ± 0.0	0.1 ± 0.1	5.0 ± 1.4	**1.0 ± 0.2**	**1.8 ± 0.1**	**2.3** ± **0.4**
T. delbrueckii	0.3 ± 0.0	0	2.1 ± 0.5	**0.5 ± 0.1**	**1.4 ± 0.0**	**1.7 ± 0.2**
Wort	1.1 ± 0.1	**221.1 ± 7.7**	34.2 ± 4.7	**14.8 ± 1.1**	**26.9 ± 1.2**	**38.6 ± 2.8**
Threshold	515 ppb	150 ppm	105 ppb	**45 ppb**	**86 ppb**	**56 ppb**

Ester production in the first fermentation had significant variation between the different yeasts, but only four values in total exceeded the threshold value (Table 8.). The values for ethylacetate were higher than the threshold value 33 mg/l with 129.5 mg/l for *C. fabianii* and 79.0 mg/l for *P. kudriavzevii.* The control strain *S. cerevisiae* produced 0.7 mg/l ethylcaproate, passing the threshold value 0.23 mg/l. Finally, the value of 2-

phenylacetate produced by *K. marxianus* was higher than the threshold of 3.8 mg/l, with 3.9 mg/l.

Table 8. Ester production in the beers after 12 °P wort fermentation at 25°C. The fermentation was ended after 143 h. Values exceeding the threshold value are highlighted in bold. Values are means from technical replicates with standard error indicating range.

Esters (mg/l)	**Ethyla-cetate**	**Ethyl-capro ate**	**Ethyl-capry-late**	**Et-hylde-canoat e**	**2-Phenylet hyla-cetate**	**3-Methyl-butyla-cetate**
C. fabianii	**129.5 ± 12.5**	**0.7 ± 0.1**	0.0 ± 0.0	0.0 **±** 0.0	0.3 ± 0.0	0.6 **±** 0.1
H. uvarum	1.0 ± 0.1	0	0	0	0	0.0 **±** 0.0
K. servazzi	9.5 ± 0.9	0	0	0	3.0 ± 0.0	0.1 ± 0.0
K. marxianus	10.1 ± 1.0	0	0	0	0.4 **±** 0.0	0.1 ± 0.0
K. marxianus	18.0 ± 1.4	0	0	0	**3.9 ± 0.2**	0.1 ± 0.0
P. fermentans	0.5 ± 0.0	0	0.0 ± 0.0	0	0	0
P. fermentans	0.3 ± 0.0	0	0.0 ± 0.0	0	0	0
P. kudriavzevii	**76.0 ± 4.3**	0	0.0 ± 0.0	0	0	0.0 ± 0.0
S. cerevisiae	25.2 ± 1.5	0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.6 ± 0.1
S. ludwigii	10.0 ± 0.7	0	0	0	0	0.0 ± 0.0
T. delbrueckii	8.0 ± 0.7	0	0	0.0 ± 0.0	0	0.0 ± 0.0
T. delbrueckii	4.5 ± 0.3	0	0.0 ± 0.0	0.0 ± 0.0	0	0.0 ± 0.0
Threshold	33	0.23	0.9	1.5	3.8	1.6

Production of higher alcohols during the first fermentation had variation between all different strains but none exceeded any of the threshold values (Table 9.). The closest measured value to the threshold value was 3-methylbutanol for *S. cerevisiae*, with 37.1

mg/l compared to the threshold value 70 mg/l. The yeasts with two strains of each had mostly similar values, except for 2-phenylethanol with *K. marxianus*, where Km1 had 3.3 mg/l while Km2 had none detected.

Table 9. Higher alcohol production in the beers after 24 h during 12 °P wort fermentation at 25°C. Values are means from technical replicates with standard error indicating range.

Aroma (mg/l)	**Acetal-dehyde**	**2-Methyl-butanol**	**2-Methyl-propanol**	**2-Phenylethanol**	**3-Methyl-butanol**	**Propanol**
C. fabianii	2.7 ± 0.3	5.8 ± 0.1	10.4 ± 0.2	3.3 ± 0.1	14.5 ± 01	5.8 ± 0.2
H. uvarum	1.8 ± 0.1	4.5 ± 0.1	15.0 ± 0.4	5.1 ± 0.1	16.3 ± 0.4	0.7 ± 0.0
K. servazzi	1.8 ± 0.2	9.8 ± 0.0	14.2 ± 0.1	0.5 ± 0.5	34.3 ± 0.0	3.9 ± 0.1
K. marxianus	0.6 ± 0.1	7.4 ± 0.1	13.2 ± 0.1	3.3 ± 0.2	19.6 ± 0.1	4.1 ± 0.1
K. marxianus	**69.8 ± 8.9**	7.5 ± 0.1	12.5 ± 0.0	0	21.4 ± 0.4	4.4 ± 0.2
P. fermentans	1.5 ± 0.2	4.3 ± 0.1	13.1 ± 0.0	5.4 ± 0.7	16.5 ± 0.1	0.6 ± 0.0
P. fermentans	1.7 ± 0.1	4.0 ± 0.0	13.8 ± 0.1	6.0 ± 0.5	17.3 ± 0.3	0.8 ± 0.0
P. kudriavzevii	3.1 ± 0.3	4.7 ± 0.1	6.0 ± 0.0	3.7 ± 0.4	13.9 ± 0.0	0.6 ± 0.0
S. cerevisiae	13.3 ± 1.1	10.3 ± 0.0	14.6 ± 0.1	9.2 ± 0.3	37.1 ± 0.1	15.0 ± 0.3
S. ludwigii	2.0 ± 0.2	5.1 ± 0.1	12.6 ± 0.1	10.0 ± 0.1	19.6 ± 0.5	3.3 ± 0.1
T. delbrueckii	3.4 ± 0.5	2.6 ± 0.1	3.4 ± 0.0	4.4 ± 0.1	20.8 ± 0.6	4.5 ± 0.1
T. delbrueckii	2.3 ± 0.2	2.6 ± 0.0	3.6 ± 0.0	2.9 ± 0.0	18.9 ± 0.1	3.8 ± 0.1
Threshold	25 mg/l	65 mg/l		125 mg/l	70 mg/l	800 mg/l

The alcohol produced in the second trial was similar to the values in the first fermentation trial, with the values measured at 143 h as 0.73% ABV for *K. servazzi*, 0.52% ABV for *P. fermentans* and 0.68% ABV for *S. ludwigii* (Fig. 6, Table 10.). Apparent attenuation rates

for the three yeasts were measured as 17.7%, 13.3% and 17.2%, respectively (Table 10.).

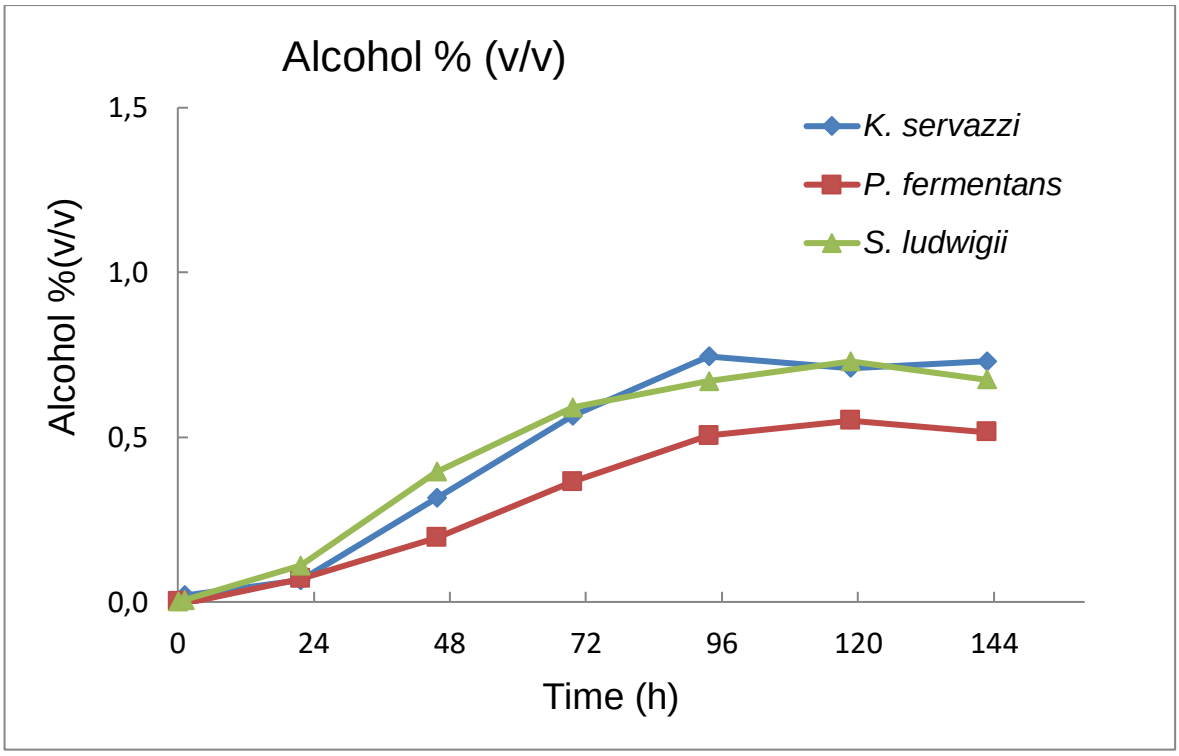

Figure 6. Alcohol content (% alcohol by volume [ABV]) in the beers fermented on 10-liter scale from 8 °P wort at 25°C. Values are means from two independent fermentations.

The pH of the worts had minimal variation and decreased from 5.14 to 4.78 at the end of the fermentation at 143 h (Fig. 7, Table 10.). The measurements were close to the 4.73 pH from the first fermentation.

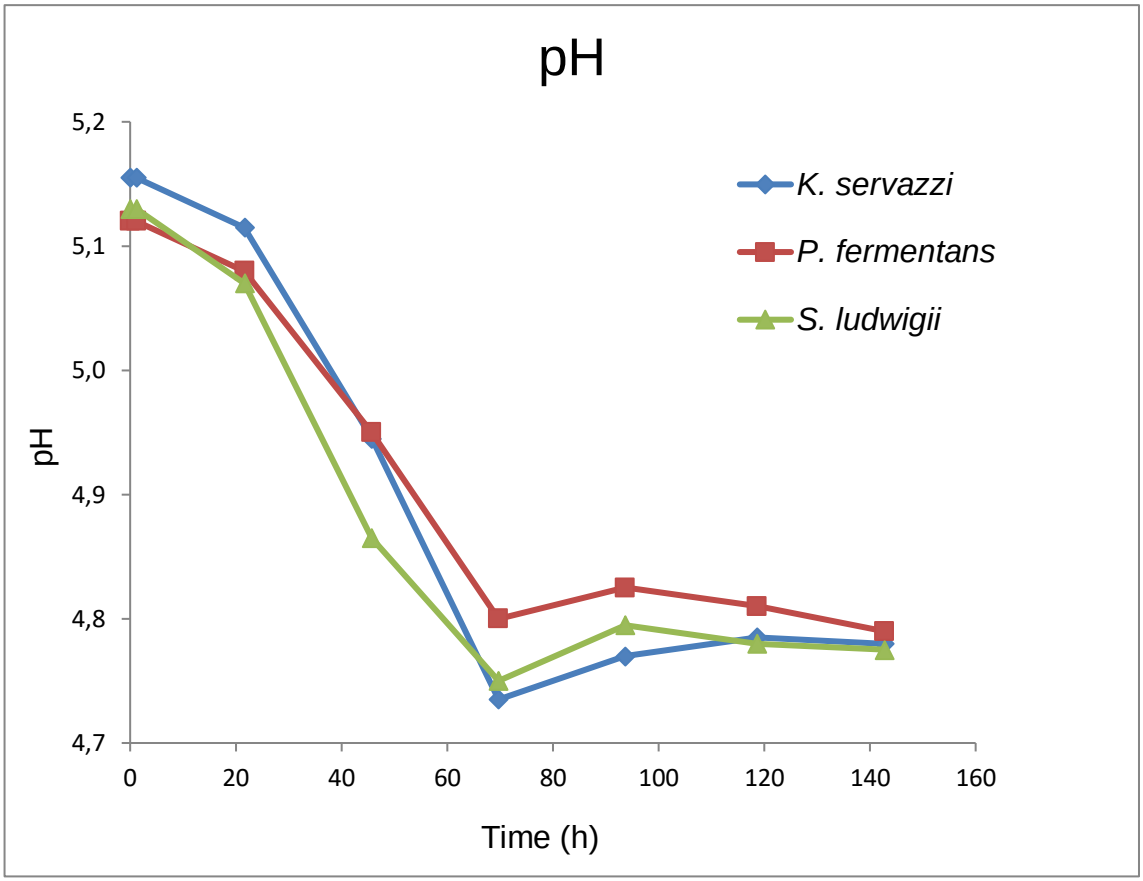

Figure 7. pH in the beers fermented on a 10-liter-scale from 8 °P wort at 25°C. Values are means from two independent fermentations.

Fermentation was ended after 143 h and the fresh mass of the yeast was measured as 4.6 g/l for *K. servazzi*, 9.5 g/l for *P. fermentans* and 6.1 g/l for *S. ludwigii*, respectively. (Table 10.). Flocculence was low in all three cases, but was highest with 27.1% for *S. ludwigii* and lowest with 0% for *P. fermentans*. Flocculence was measured as 4.0% for *K. servazzi* (Table 10.).

Table 10. Values of the green beer after 143 h of fermentation and flocculence. Flocculence was evaluated by following the procedure of the Helm assay. The values are means from two independent replicates with standard error indicating range.

Values of green beer	***K. servazzi***	***P. fermentans***	***S. ludwigii***
Alcohol % (v/v)	0,73 ± 0,0	0,52 ± 0,0	0,68 ± 0,0
pH	4,78 ± 0,0	4,79 ± 0,0	4,78 ± 0,0
Apparent attenuation %	17,7 ± 0,1	13,3 ± 0,0	17,2 ± 0,1
Yeast fresh mass (g/l)	4,6 ± 0,0	9,5 ± 1,5	6,1 ± 0,5
Viability %	90,3 ± 0,1	96,3 ± 0,2	93,5 ± 0,0
Helm assay			
Flocculence %	4,0	0	27,1

Some compounds in the second fermentation had a similar production of aroma volatiles in comparison to the first fermentation trial, while others varied by up to a tenfold. Aroma production was overall low, with only two values exceeding the threshold value (Table 11.). The values for 2-phenylethylacetate was higher than the threshold value of 3.8 mg/l with 7.4 mg/l for *K. servazzi* and 6.1 mg/l for *S. ludwigii*. In the first fermentation, 2-phenylethylacetate was below the threshold value for K. servazzi with the value 3.0 mg/l. The compounds with noticeable differences from the first fermentation trial was ethylacetate, 2-phenylethylacetate and 2-methylpropanol. Ethylacetate was measured for the yeast *S. ludwigii* as 10.0 mg/l in the first trial and 1.3 mg/l in the second. 2-phenylethylacetate was 0 mg/l in the first fermentation for *S. ludwigii,* comparing to 6.1 mg/l in the second. 2-methylpropanol was measured for *P. fermentans* as 13.8 mg/l in the first fermentation trial and 4.9 mg/l in the second.

Table 11. Acetaldehyde and aroma compounds produced during 8 °P wort fermentation with threshold values. Values are means from technical replicates with standard error indicating range.

	K. servazzi	***P. fermentans***	***S. ludwigii***	**Threshold value**
Acetaldehyde (mg/l)	3.8 ± 0.1	2.3 ± 0.0	2.8 ± 0.4	25
Aroma (mg/l)				
Ethylacetate	0.8 ± 0.0	0	1.3 ± 1.0	33
Ethylcaproate	0	0	0	0.23
Ethylcaprylate	0	0	0	0.9
Ethyldecanoate	0	0	0	1.5
2-Methylbutanol	4.1 ± 0.1	2.1 ± 0.0	5.6 ± 0.0	65
2-Methylpropanol	7.5 ± 0.2	4.9 ± 0.1	8.7 ± 0.2	
2-Phenylethanol	0	2.8 ± 0.1	0	125
2-Phenylethylacetate	**7.4 ± 0.2**	0	**6.1 ± 0.4**	3.8
3-Methylbutanol	17.0 ± 0.3	8.7 ± 0.2	23.7 ± 0.1	70
3-Methylbutylacetate	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	1.6
Propanol	2.1 ± 0.1	0.3 ± 0.0	1.2 ± 0.1	800

Only the aroma was measured from the second fermentation. Measurements of aldehydes and higher alcohols from the second fermentation were seen as unnecessary, as values were not predicted to differ to a large degree and aroma was the primary focus.

5 Discussion

In this study, ten yeasts isolated from diverse *de novo* sourdough cultures were investigated. The yeasts were selected from a variety of isolates with potentially interesting aroma and other characteristics relevant for brewing. Previous studies into the brewing potential of sourdough-derived yeasts have primarily included *S. cerevisiae* yeasts (Catallo et al. 2020; Marongiu et al. 2015; Mascia et al. 2015; Ripari et al. 2018; Rossi et al. 2018). This study is the first to examine non-*Saccharomyces* yeasts isolated from sourdoughs for the purpose of brewing.

This approach proved to be beneficial, with screening being effective in the identification of yeasts with low-alcohol brewing potential. Screening included the use of spot plates, which was straightforward and yielded clear results. Yeasts growing at 37°C have a risk of being pathogens and this should be considered when determining the suitability for use. Yeasts growing at 15°C may be beneficial for the brewing environment as many brewing processes are conducted at low temperatures. The advantage of a lower ethanol tolerance is easier handling in the brewery, equipment and surfaces can effectively be disinfected and there is a significantly lower risk of cross contamination. The yeast *K. servazzi* had high sensitivity to ethanol. Yeasts *C. fabianii*, *P. kudriavzevii*, *S. ludwigii* and *T. delbrueckii* had minimal growth at incubation temperature 15°C. *K. marxianus* and *P. kudriavzevii* preferred 37°C and grew more rapidly than at 25°C, which likely makes them unsuitable for brewing purposes due to potential health risks. The potential pathogenicity of *P. kudriavzevii* has been highlighted recently (Douglass et al. 2018). The three strains selected for the second fermentation, *K. servazzi*, *P. fermentans* and the reference yeast *S. ludwigii*, were all unable to grow at 37°C. This minimizes their risk of being pathogens. The relative cold tolerance of *K. servazzi* in comparison to the reference *S. ludwigii* could have benefits if applied to industrial use.

Determining phenolic flavour notes allows differentiation and selection of strains for different brewing purposes. There are currently no commercially available strains for production of low-alcohol beers with phenolic flavour notes. Out of the three strains selected for the second fermentation, *P. fermentans* (Pf2) was POF^+ while *K. servazzi* and *S. ludwigii* were POF^-. The phenolic notes produced by *P. fermentans* would make it

suitable for use in the production of low-alcohol wheat beer, or any other beer style where this flavour is typical.

First fermentation trial showed, like expected, that all tested strains produced low levels of alcohol due to an inability to ferment the sugar maltose in the wort. The results show that most of the non-conventional yeasts are capable of aldehyde reduction and could be used to remove the raw 'worty' flavour notes typical of many low-alcohol beers. Sensory analysis is an important part of evaluating the suitability of a yeast for commercial use. Production of aromatic compounds were overall low, but there was potential in some of the studied isolates. Two yeasts, *K. servazzi*, *P. fermentans* and the reference yeast *S. ludwigii* were selected for a second fermentation based on limited prior published studies, presumed safety, lack of phenolic off-flavours and the phenylethyl acetate production in *K. servazzi.* The phenylethyl acetate production was higher in the 10L-scale fermentation, this time above the threshold value.

Studies into the brewing application of sourdough-derived yeasts excluding *S. cerevisiae* have been limited. However, studies into some of the yeasts included in this thesis have been conducted. These previous studies have included isolates from different sources. Previous studies into non-conventional yeasts have shown relatively low production of overall aromatic compounds but some have shown potential for low-alcohol brewing. Studied yeasts have produced some pleasant fruity or floral aroma. Studies into *T. delbrueckii* include strains isolated from beet sugar juice. A low concentration of secondary metabolites was concluded in one study. Sensory analysis revealed honey and pear-like character and other citrus fruit-like character (Bellut and Arendt 2019; Holt et al. 2018; Michel et al. 2016).

A study conducted by van Rijswijck et al. included wild *C. fabianii* and *P. kudriavzevii* isolates fermented in co-cultures with wild *S. cerevisiae* yeasts. The *C. fabianii* and *P. kudriavzevii* isolates produced relatively more esters compared to the *S. cerevisiae* isolates despite their limited fermentation efficiency. The *P. kudriavzevii* isolates had a final ABV of 0.5% - 0.8%, while the *C. fabianii* reached 0.6% ABV. A further study into ester production of *C. fabianii* and *P. kudriavzevii* isolates in co-cultures with *S. cerevisiae* was conducted (van Rijswijck et al. 2017; 2019).

Holt et al. conducted a study including several non-*Saccharomyces* yeasts from various sources, such as two *P. kudriavzevii* isolates from cacao fermentation and ginger beer. The isolates produced high levels of ethyl acetate and formed isoamyl alcohol exceeding the threshold value (Bellut and Arendt 2019; Holt et al. 2018).

Some of the previous studies demonstrate similar results to those of the current study, such as low ethanol production, low overall aromatic compounds but with some exceptions that show potential for brewing purposes. Strains producing aromatic compounds in a higher concentration had pleasant floral or fruity aromas.

6 Conclusions

The aim of the thesis was to study sourdough yeasts and their potential suitability for low-alcohol beer. On the basis of the results of the study, sourdough yeasts can be seen as a viable option for the purpose of low-alcohol brewing.

Sourdoughs have a complex ecology consisting of a broad range of yeasts and lactic acid bacteria forming a mutual association that plays a key role in the production of sourdough. This mutual association makes the fermentation process in the dough possible. Due to the high yeast diversity, availability and presumption of safety for consumption, sourdough can be considered as a viable source of new yeasts.

Ten yeasts isolated from sourdoughs were selected for screening and pilot-scale fermentation trials. Further characterization revealed that some yeasts were unsuitable for this application due to off-flavour production, inability to produce positive flavour notes at a relevant concentration or poor tolerance to conditions relevant to brewing. The yeast strains *K. servazzi* and *P. fermentans* were uniquely suited for the production of low-alcohol beers and performed as well as the reference strain *S. ludwigii*. The yeast *K. servazzi* produced a notable amount of the ester 2-phenylethylacetate, which has a pleasant floral aroma. The relative cold tolerance of *K. servazzi* in comparison to the reference *S. ludwigii* could have benefits if applied to industrial use.

This thesis demonstrated the need for more studies into sourdough-derived yeasts for brewing purposes and the need for further investigation of the work done during this thesis. Further study would require more pilot-scale and industrial-scale fermentations, joined by appropriate sensory analyses of the final beers to demonstrate the full potential of these strains. A research article based on the results of this thesis will be submitted in the near future.

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Appendix 1. Sequenced yeasts

Table 12. Yeast strains identified by sequencing. Strains highlighted in bold were used in the experiments.

Code	Identification	% ID	Fragment size (bp)	Sourdough
1a 10-3 A2	*Pichia fermentans*	100	450	Whole Grain Graham Wheat
1a 10-3 B1	*Pichia kudriavzevii*	100	500	Whole Grain Graham Wheat
1a 10-3 B2	***Pichia fermentans***	100	450	Whole Grain Graham Wheat
1a 10-3 B3	*Pichia fermentans*	96	400	Whole Grain Graham Wheat
1a 10-4 B1	*Pichia fermentans*	96	700	Whole Grain Graham Wheat
1a 10-4 B2	***Hanseniaspora uvarum***	100	600	Whole Grain Graham Wheat
1a 10-4 A	*Pichia fermentans*	99	750	Whole Grain Graham Wheat
1b 10-4 A1	*Pichia fermentans*	99	450	Whole Grain Graham Wheat
1b 10-4 B1	*Kluyveromyces marxianus*	100	650	Whole Grain Graham Wheat
1b 10-4 B2	*Pichia fermentans*	99	400	Whole Grain Graham Wheat
1b 10-4 B5	*Hyphopichia burtonii*	99	400	Whole Grain Graham Wheat
3b 10-3 A1	*Hyphopichia burtonii*	99	400	Wheat
3b 10-3 A2	*Hyphopichia burtonii*	99	400	Wheat
3b 10-3 B3	*Wickerhamomyces anomalus*	100	550	Wheat
4a 10-3 A1	*Wickerhamomyces anomalus*	100	550	Wheat

4a 10-3 A2	*Wickerhamomyces anomalus*	100	550	Wheat
4b 10-2 A3	*Wickerhamomyces anomalus*	100	600	Wheat
4b 10-2 B1	*Wickerhamomyces anomalus*	100	600	Wheat
5a 10-3 B1	***Torulaspora delbrueckii***	99	700	Organic Rye
5a 10-3 2	*Torulaspora delbrueckii*	99	700	Organic Rye
5a 10-3 3	*Torulaspora delbrueckii*	99	700	Organic Rye
6a 10-3 A3	*Cyberlindnera fabianii*	99	600	Rye
6a 10-3 B1	***Pichia fermentans***	99	450	Rye
6a 10-4 A2	*Pichia kudriavzevii*	98	500	Rye
6b 10-4 B1	*Pichia kudriavzevii*	88	700	Rye
6b 10-4 B2	*Pichia kudriavzevii*	99	500	Rye
6b 10-4 B15	***Kluyveromyces marxianus***	99	700	Rye
7a 10-4 A2	*Pichia kudriavzevii*	98	500	Rye malt
7a 10-4 A3	***Kazachstania servazzi***	99	700	Rye malt
7a 10-4 A4	*Cyberlindnera fabianii*	100	600	Rye malt
7a 10-4 B1	*Pichia kudriavzevii*	99	500	Rye malt
7b 10-4 A3	***Cyberlindnera fabianii***	99	600	Rye malt
7b 10-4 B3	*Wickerhamomyces ciferrii*	77	600	Rye malt
8b 10-4 A6	*Torulaspora delbrueckii*	100	600	Rye malt
8b 10-4 B1	*Pichia kudriavzevii*	98	800	Rye malt
8b 10-4 A13	***Torulaspora delbrueckii***	99	700	Rye Malt
8b 10-4 A14	***Pichia kudriavzevii***	99	900	Rye Malt
9a 10-3 B2	*Wickerhamomyces anomalus*	99	500	Barley
9a 10-3 B3	*Wickerhamomyces anomalus*	99	600	Barley
9b 10-3 A1	*Pichia kudriavzevii*	97	600	Barley

9b 10-3 A2	***Kluyveromyces marxianus***	100	700	Barley
9b 10-3 A3	*Wickerhamomyces anomalus*	79	700	Barley