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Ronja Eerikäinen

Assessing Contamination Risks of Non-Conventional Yeasts in Breweries

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The Technical Research Centre of Finland Ltd commissioned this Bachelor Thesis. The main goal of this thesis was to examine the safety of certain yeast strains in beer production and how to prevent contamination or proliferation. Different types of yeast make it possible to develop new beer styles and flavour profiles, which is why further research into them, for example, in terms of safety, is essential.

The thesis is based on, among other things, VTT's pilot brewery's previous findings on contamination caused by the *Kazachstania servazzi* strain. For this reason, a more detailed study of different yeast strains was considered necessary, and more information was needed on their behaviour under other conditions.

In this Bachelor's thesis project, 25 different yeasts were studied, most of which are non-conventional yeasts as well as known contaminants in the food and beverage industry. The study was carried out in VTT's brewery's laboratory, where biofilm production, disinfectant tolerance, preservative tolerance and the ability of yeasts to grow at different temperatures were tested. When new yeasts are used, their use and development should be observed, and adequate hygienic conditions in production should be ensured.

In the Bachelor thesis, it was found that most of the yeasts studied could be safe for brewing based on the results. The study showed that *K. servazzi* was the most challenging yeast strain to control out of the tested yeast strains, and its use in beer production appears to involve the most significant risks.

Further research is needed especially for those yeasts that produced a large amount of biofilm in the experiments. In further research, it would be good to test their tolerance, for example, against cleaning agents. Also, further research should be used to determine the clinical importance of some yeasts with, for example, antibiotic tests.

Keywords	non-conventional, yeast, beer, NABLAB, brewery, wild yeast, contamination

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Tämä insinöörityö tehtiin Teknologian tutkimuskeskus VTT Oy:n toimeksiantona. Työn tärkeimpänä tavoitteena oli selvittää tiettyjen hiivalajikkeiden turvallisuutta oluen valmistuksessa sekä sitä, kuinka näistä lajikkeista tarvittaessa päästään eroon mahdollisen kontaminaation sattuessa. Eri hiivalajikkeet mahdollistavat uusien olutmakujen kehittämisen ja tästä syystä niiden lisätutkimus muun muassa turvallisuuden osalta on tärkeää.

Tutkimuksen taustalla on muun muassa VTT:n pilottipanimon aiemmat havainnot *Kazachstania servazzi*-kannan aiheuttamista kontaminaatioista. Tästä syystä eri hiivalajikkeiden tarkemmalle tutkimiselle nähtiin tarvetta ja niiden käyttäytymisestä eri olosuhteissa haluttiin lisätietoa.

Tässä insinöörityössä tutkittiin 25:tä eri hiivaa, joista suurin osa on villihiivoja sekä tunnettuja kontaminantteja ruoka- ja juomateollisuudessa. Tutkimus toteutettiin VTT:n panimon laboratoriossa, jossa testattiin biofilmin tuotantoa, desinfiointiainetoleranssia, säilöntäaineiden sietokykyä sekä hiivojen kykyä kasvaa eri lämpötiloissa. Käytettäessä uusia hiivoja tulisi niiden käytöstä ja kehitystä tarkkailla sekä varmistua riittävän hygieenisistä olosuhteista tuotannossa.

Insinöörityössä huomattiin, että suurin osa tutkituista hiivoista voisi tulosten perusteella olla turvallisia oluen panemiseen. Tutkimuksessa kävi ilmi, että testatuista hiivalajikkeista *K. servazzi* oli kaikkein hankalimmin hallittavissa oleva hiivakanta ja sen käyttö oluen valmistuksessa vaikuttaisi sisältävän suurimmat riskit.

Jatkotutkimusta tarvittaisiin etenkin niille hiivoille jotka tuottivat kokeissa paljon biofilmiä. Jatkokokeissa olisi hyvä testata niiden sietokykyä mm. puhdistusaineille. Lisäksi tulisi jatkotutkimuksen avulla selvittää joidenkin hiivojen kliininen merkitys esimerkiksi antibioottitesteillä.

Avainsanat	villihiiva, olut, alkoholiton, kontaminaatio, panimo, hygienia

Contents

List of Abbreviations

ABV	Alcohol by volume. A standard measure of how much alcohol is contained in a given volume of an alcoholic beverage.
ADI	Acceptable Daily Intake. Means the maximum acceptable daily intake for a food additive, drug or pesticide, which can be exposed to each day without adverse effects.
CIP	Clean in place. A method of cleaning the interior surfaces of equipment's, filters, pipes, vessels and associated fittings, without major disassembly.
EFSA	The European Food Safety Authority. An authority that provides independent scientific advice and communicates on existing and emerging risks associated with the food chain.
EPS	Extracellular Polymeric Substance. Natural polymers of high molecular weight secreted by microorganisms into their environment.
FAO	Food and Agriculture Organization. A specialized agency of the United Nations that leads international efforts to defeat hunger and improve nutrition and food security.
GABA	γ-aminobutyric acid. The main inhibitory neurotransmitter in the developmentally mature mammalian central nervous system.
HACCP	The Hazard Analysis and Critical Control Points. A management system in which food safety is addressed through the control and analysis of physical, chemical and biological hazards from raw material production to manufacturing, and to the finished product.
IBU	The International Bittering Units scale. A scale to approximately quantify the bitterness of beer.
JECFA	Expert Committee on Food Additives. An international scientific expert committee administered jointly by the FAO and WHO.

NABLAB Non-alcoholic beer and low alcoholic beer.

QS Quorum sensing. The ability to detect and to respond to cell population density by gene regulation.

1 Introduction

VTT, the Technical Research Center of Finland Ltd commissioned this final project in engineering. VTT is a research and development organization that produces innovative solutions for companies and the public sector.

The popularity of non-alcoholic and low-alcohol beers is on the rise. However, the general idea of non-alcoholic beers is that they are a tasteless and watery version of regular beer, and in many cases, this is very true. [1.] Alcohol can be removed from beer in many different ways, such as filtration and distillation or limited fermentation. In each case, the greatest challenge is to reduce the amount of alcohol without losing the taste of the beer. [2.] Traditional brewer's yeasts (*Saccharomyces cerevisiae* (ale) and *S. pastorianus* (lager)) are effective in using wort sugar alcohol, which is problematic in the production of non-alcoholic beers because many of the essential flavour components of beer result from yeast metabolism. Therefore, the search for alternative methods for the production of non-alcoholic beers has begun. [2; 3.] Wild yeasts have been studied in beer production for some time. They have been, for example, sources of contamination in breweries, but in some cases, they have been found to bring new good flavours to beers. [3.] VTT began researching wild yeasts around 2015. To use wild yeasts, one should be sure of their safety and be able to manage them so that they do not cause problems in production.

In this final project in engineering, 25 different yeasts were studied, most of which are non-conventional yeasts as well as known contaminants in the food and beverage industry. The study aimed to find whether these yeasts would be safe to use in brewing and how they could be managed. Some yeasts have already shown potential in alternative methods of making different beers. Traditional brewer's yeasts were also included to compare the differences between them and non-conventional yeasts. The production of yeast biofilm, disinfectant resistance, tolerance of different preservatives and the ability to grow at different temperatures were studied. All these properties affect the processing of yeasts in production. The better they produce biofilm, grow at different temperatures,

and tolerate preservatives as well as disinfectants, the more challenging they become in managing potential contamination. Without adequate knowledge of hygiene principles, contamination management is not possible. [4, p. 366.] The theoretical part of the thesis deals with the hygiene management methods needed in the food industry.

The experiments were performed in the laboratory of VTT's brewery. Biofilm production was tested by growing yeasts from one to five days in microplate culture, and the formation potential was assessed by measuring cell adhesion to the well's walls. Disinfectant tolerance was tested on YPD agar plates under clean and unclean conditions at different exposure times. The tolerance of the preservatives was tested in microplate cultures grown in a Bioscreen incubator for three to five days. The ability of yeasts to grow at 1, 4 and 37 degrees was tested on YPD agar plate cultures for three days to three weeks.

2 Non-alcoholic beer and low alcoholic beer (NABLAB)

NABLAB is an abbreviation of two different beer types. NAB means non-alcohol beer and has an alcohol by volume (ABV) of ≤ 0.5 %. LAB means low-alcohol beer and ABV is between 0.6 - 3.5 %. NABLAB beers have different names like ‘’small beer’’, ‘’near beer’’ and ‘’light beer’’, for example. [1.]

2.1 Trends and health

There is a growing interest in novel and traditional beer brewing [1]. This has coincided with a growing interest in health and well-being. The raised awareness of the ills caused by alcohol and people's own preferences have inspired a search for alternatives to alcoholic beverages. [5.] Alcohol is known to cause many ailments such as reduced sleep quality, intestinal problems, increased risk of various cancers, and can aggravate mental health problems such as depression and anxiety [6].

The health effects of drinking non-alcoholic beer have not been researched largely. Non-alcoholic beer may have some sedative effects, such as better sleep quality and decreased anxiety, probably due to the presence of hop in beer. Studies have shown that the hop increases the activity of the neurotransmitter ɣ-aminobutyric acid (GABA). As GABA levels rise in the brain, nervous activity decreases. Hops contain bitter acids, myrsenol- and xanthohumol compounds. Hops xanthohumol polyphenol is believed to have a positive effect on cardiovascular health. [5; 7, p. 140-142.]

Alcohol-containing products have a significantly higher calorie content than their non-alcohol counterparts [8]. Beer itself contains good nutrients such as B vitamins and minerals. In non-alcoholic beer the health benefits are available without the disadvantages of alcohol [1]. Finnish alcohol culture pays more attention to responsible drinking nowadays. An example of this is the Välivesi-product launched by Alko. [9].

The importance of social eating and drinking has increased and so has the visual appearance of the meals. Due to social media, people pay more attention to how they appear. People want to share thoughtful and successful images and healthy lifestyles. The alcohol culture at parties has also changed. The purpose is no longer to drink alcohol to the fullest, but rather to enjoy good food and company. Experience and excitement is no longer sought only from alcohol. [10.]

2.2 Market at the moment

The popularity of non-alcoholic beverages has increased significantly in the recent years. It is still popular to drink beer, but because of its taste, not because of its intoxicating effects. This is why many large companies now make and develop new non-alcoholic beers according to demand. [11.] Small brewers have also shown interest in non-alcoholic beer, but so far their production is still relatively low [1].

2.3 Growth expectations

From 2011 to 2016, NABLAB's global market grew by 20 % and demand is expected to grow by 24 % by 2021. In Germany, 400 different non-alcoholic beers received 6 % of the country's total beer sales in 2017. The assumptions to the taste of non-alcoholic beer also varies from country to country. In France 50 % of beer drinkers think that non-alcoholic beer does not taste as good as standard beer, while in Germany this figure is only 28 %. If the taste of the non-alcoholic beer would be as good as standard beer, 50-65 % of Europeans would choose the non-alcoholic beverage. [1.]

In Finland, sales of non-alcoholic beers are clearly growing. Growth was 20 % from 2015 to 2018 and at the beginning of 2019 it was up to 35 % compared to the same period last year. [12.]

There are bars in, for example, New York and Dublin that only serve non-alcoholic drinks. Such bars are not expected to arrive in Finland any time soon, but the function is tested by focusing on non-alcoholic beverages so that alcoholic beverages are also on offer. Non-alcoholic beverages are part of the well-being trend, and it is believed that this phenomenon is ongoing. [13.]

3 Brewery

3.1 Traditional brewing process

Beer has been brewed for thousands of years. In 1516 in Bavaria, the famous law known as “Rheinhaitsgebot” ordered that only water, barley and hops could be used as ingredients for brewing. With the addition of yeast, these ingredients still form the base for brewing. The role of yeast in fermentation was shown by Louis Pasteur in the 19th century and after Carlsberg started pure-cultured yeast fermentations in 1883, there have not been major changes in the brewing process. [14.]

Prior to brewing, barley is malted. This is usually done in malt-houses outside the brewery. First, the barley is soaked in water for 48 hours at 14-18 °C to reach a moisture content of 42-46 % which allows the grains to germinate. Germination takes 3-5 days at 16-20 °C. Enzymes break down cell walls and some of the proteins in the starchy endosperm, which makes the grain crumble and easier to grind. During germination, amylases are produced, which are important for the degradation of starch during the mash. Visible signs of malting are softening of the grain, growth of acrospires underneath the husk, and development of roots. The kilning of the malt takes place gradually, at which point the germination stops. The temperature is raised to 50-110 °C to reach a moisture content of < 5 %, while retaining activity of heat-sensitive enzymes. The more intense the kilning process is, the more roasted and burnt its flavor characteristics and the darker the malt. [15, p. 4.]

In the brewery, the malted grains are milled, after which they are mixed with hot water. The water must contain salts and the salt profile of the water may have an effect on the outcome of the beer. Famous pilsners, for example, are made from water that contains only a small amount of calcium, while some high-quality ale beers are brewed with water with a high calcium content. The mash typically contains three parts water and one part malt. Some breweries supplement the malt bill by adding, for example, corn or rice. [15, p. 4.] Temperature range in mashing can be from 30 to 78 °C. The temperature is used to control the activity of the enzymes; thus, the desired hydrolysis level is achieved. The primary enzymatic reactions are dissolving and breaking the starch into fermentable and non-fermentable sugars, and solubilizing and breaking proteins. [16, p. 77.]

After about an hour of mashing, the liquid phase of the mash, i.e., the wort, is removed. The wort is recovered either by filtration through spent grains or through plates. The wort is run into a kettle and boiled for about an hour. Boiling sterilizes the wort, removes the granular characters of the barley, and precipitates proteins that would otherwise produce turbidity in the beer. At this point, most breweries add hops, and some breweries may also add extra sugars. Hops have two important components, essential oils and resins. Resins, or α-acids, are isomerized during brewing to iso-α-acids. Iso-α-acids give the

beer its bitter taste. This process is relatively inefficient and hops today are often extracted with liquefied carbon dioxide at low temperatures. They are added to a kettle or added to the finished beer in isomerized form. This also avoids the adhesion of bitter substances to the yeast. [15, p. 4-5.]

The characteristic hop aroma of beer is derived from hop oils. Due to their volatile nature, the aroma can be lost if the hops are added too early in the boiling step. Thus, aroma hops are usually added towards the end of the boiling, so-called late hopping, leaving more hop aroma in the wort. To ensure that none of volatiles are lost due to boiling, part of the hops can be added directly into the fermenters at the end of the process (so-called dry hopping). Dry hopping enables the characteristic nature of these products due to the oil mixtures. Extraction of oils and resins with liquid carbon dioxide also allows the extracts to be used in the late stages of the process to change the taste of the beer. [15, p. 5.]

The precipitates formed during boiling are removed and the hopped wort is cooled, aerated and yeast is added. Brewer's yeasts can be divided into bottom-fermenting yeasts (*Saccharomyces pastorianus*) and top-fermenting yeasts (*S. cerevisiae*). Fermentation with bottom-fermenting yeast can take several weeks and their temperatures are low, for example, from 6 to 15 °C. Top-fermenting yeasts are faster and fermentation can be completed in just a few days. Temperature ranges vary from 18 to above 20 °C. Both need a little oxygen to start their metabolism, but otherwise alcoholic fermentation is anaerobic. [15, p. 5-7.] During the fermentation, the fermentable sugars are converted into ethanol and carbon dioxide and most of the essential aroma and flavor compounds are formed as by-products of the yeast metabolism. [17, p. 5]. Once the desired alcohol content is achieved, the primary fermentation is ended, and the yeast can be collected for the next fermentations. Beer is the only alcoholic beverage that recycles yeast in this way. [15, p .5-7.]

From the primary fermentation, the beer goes into secondary fermentation, in which, the beer is matured and clarified. Its preservation properties and taste aromas can be changed. During the secondary fermentation, the diacetyl – a compound which has a

distinctive butterscotch taste - is removed by yeast cells still present in the beer. The more diacetyl there is after the primary fermentation, the more effective and longer storage the beer requires. The diacetyl content can be considered as a measure of beer maturation. [16, p. 129.] The beer is packaged after either sterile filtration or pasteurization. Small packages such as cans and bottles can be pasteurized within the package. [17, p. 5.]

Although brewing is mainly the same everywhere, there are no two identical breweries. The working and appearance of the equipment will vary depending on the premises and available equipment's and the preferences of the brew master. [18, p. 10.]

3.2 NABLAB brewing process

In general, the non-alcoholic and low-alcoholic beers are similar to normal beers except for the lack of alcohol. Thus, their production comprises the same steps from malting to mashing and to fermentation. The tricky part is how to restrict alcohol content without affecting the taste too much. There are several methods to remove alcohol in the production of non-alcoholic beers. For example, dilution procedure, fermentation-free brewing and alcohol removal/dealcoholization and restricted alcohol fermentation method. [19.]

In alcohol removal (dealcoholization) process, alcohol is removed from the beer during the fermentation by different methods. The alcohol removal process includes heat- and membrane-based processes, such as dialysis, reverse osmosis, vacuum distillation and water vapour stripping under vacuum. These methods may cause variations in quality parameters such as acidity, colour, flavour, fragrance and stability. [19.]

In cold-contact fermentation, wort is fermented at 0 to 8 °C for 24-100 hours. Wort pH is suggested to be adjusted to 4. During the limited fermentation in anaerobic conditions at low temperatures, cells produce only the desirable beer flavours and reduce wort off-flavours. Aldehydes, such as heptanal, hexanal, 2- and 3-methylbutanal, which are re-

lated to worty off-flavour, are reduced to corresponding higher alcohols. Also, intracellular aldehydes, formed as intermediates in the cell metabolism, are reduced to fusel alcohols, for example, iso-amyl alcohol, isobutanol and 1-propanol. [19; 20.]

In the restricted fermentation method, the production of alcohol is reduced in the early stages of fermentation. This is achieved either by interrupting or repressing fermentation by applying different compositional and/or process procedures (interrupted fermentation technique) or using yeast that can only partially ferment the wort. Using yeasts helps to impart a beer taste. Even if yeasts are not physiologically capable of producing a full alcoholic fermentation, they may still release flavour-active metabolites under these conditions. An advantage compared to other methods is that the dealcoholization stage and its difficulties are avoided. Especially from a flavour perspective, the resulting beers are usually organoleptically more acceptable than those obtained by dealcoholization procedures. However, the process has not been entirely sufficient from an organoleptic point of view, as the drinks differ to a fairly large degree from regular beer and have a more or less “artificially-flavored-beer” taste. [19.]

3.3 Alternative ways of producing beers

Because the customers’ demand for diversity in beer styles is increasing, the search for new approaches, such as the use of alternative yeasts, is relevant nowadays. Different ways to produce alternative beers are, for example, spontaneous fermentation, light beer and using non-conventional yeasts. [2.]

In spontaneous fermentation, brewery-resident microorganisms have driven the process. Most common spontaneous fermentation beers are the lambic beers of Belgium. In the production of lambic beer, the wort is inoculated during the overnight cooling performed in thin tanks. Then the wort is moved into the wooden barrels, where it is submitted to fermentation and ageing. Several yeasts and bacterial species develop during the fermentative process. The microbial consortium varies relatively little between different fermentation batches, which indicates that in each brewery there is a resident microbiota.

This allows increasing process reproducibility among successive fermentations. Microbial autolysis is most likely the influence of the unique flavour profile of spontaneous fermentation beers. In this thesis, the known contaminating yeasts *Brettanomyces* spp., especially *B. bruxellensis*, have brought a positive acetic acid flavour to lambic beers. [2.]

Due to obesity problems, especially in western populations, low-calorie beers have accounted for a growing market segment. Low-calorie beers can be achieved by alternative production methods, mainly reduction of carbohydrates in the final product. The used process is the “dilution” of wort carbohydrates, which unfortunately leads to low-flavour and poorly structured beers. Another technique is during the mashing a conversion of higher wort sugars, which determines fermentation a high attenuation level, thus lowering the content of sugar in the final product. [2.]

Barley, malt and hops are responsible for some of the aroma compounds in beer. However, the leading players that influence the aroma and flavour are the yeasts. Thus, yeasts are not only for good fermentation yield-efficiency. The primary by-products formed during fermentation are ethanol and CO_2. Other yeast-derived flavour compounds are carbonyls (aldehydes/ketones), esters, fatty and organic acids and sulphur compounds. Carbohydrates and nitrogenous compounds are the two key nutrient classes that influence brewing yeast performances. Brewing strains can use several carbohydrates such as glucose, sucrose, fructose, maltose, galactose, raffinose and maltotriose. [2; 3.]

Non-domesticated non-*Saccharomyces* and wild yeast strains can provide different flavour and aroma characteristics potentially resulting in new beer styles and variations. A natural alternative way to bring innovation to affecting the overall organoleptic profile of beers is the use of non-*Saccharomyces* yeasts, which allows finding a diversity of aroma and flavour volatiles. It is generally assumed that non-*Saccharomyces* yeasts have adverse effects on beer production, such as beer filterability, sourness, turbidity, viscosity, phenolic off-flavours and other flavour profile changes. Compared to *S. cerevisiae*, non-conventional yeast species generally have lower performance for ethanol production.

They have only been rarely used as pure starter cultures for the production of alcoholic beverages. In contrast, they are often used during co- or sequential fermentations with *S. cerevisiae*. [2; 3.]

Some of the non-conventional yeasts used in this project have also been tested and have had good effects on the production of innovative beers (Table 1). [2.]

Table 1. Non-conventional yeasts for production of innovative beers. Different yeasts species used for bioflavouring, alcohol reduction and calories reduction [2].

Bioflavouring	**Alcohol Reduction**	**Calories Reduction**
Brettanomyces: ester production (fruity or floral character); -glucosidase activity	*Pichia kluyveri*: limited ability to ferment glucose	*Brettanomyces*: β-glucosidase activity (degradation of dextrins)
Lachancea thermotolerans: production of lactic acid (flavor and mouthfeel)	*Saccharomycodes ludwigii*: low fermentation activity in maltose and maltotriose	*Saccharomyces cerevisiae var.diastaticus*: glucoamylase activity (digestion of dextrins)
Torulaspora delbrueckii: production of 2-phenylethanol and amyl alcohols (fruity and floral aroma)	*Torulaspora delbrueckii*: inability to ferment maltose and maltotriose	
Wickerhamomyces anomalus: production of ethyl acetate, ethyl propanoate, phenyl ethanol, and 2-phenylethyl acetate (fruity aroma)	*Zygosaccharomyces rouxii*: total or partial inability to ferment maltose	

A useful alternative to produce low/non-alcohol beers is to use yeasts that have limited ability to use wort sugars, i.e., maltose-negative yeasts. These species, may however, produce typical concentrations of flavour compounds and retain some of the aromatic complexity of conventional beers. In non- and low-alcohol beers made by stopped fer-

mentation there often occurs a "worty" taste. Such yeasts can also reduce wort aldehydes and thus eliminate this taste. The successful use of non-conventional yeasts in brewing is the handling of these new yeasts in a controlled manner to reach the desired beer characteristics. [2.]

4 Hygiene management

4.1 Contamination management and risks in production

Ensuring the microbiological quality of food requires managing the microbiology of the entire production chain. Yeasts are mainly known as beneficial organisms in the production of beer, bread and wine, but they can also have adverse effects on food. Uncontrolled yeast growth at different stages of the production chain can lead to a deterioration in the organoleptic quality of the products and production problems and thus economic losses. [21.] Contamination management is based on prevention and cleaning of premises and equipment. Without adequate knowledge of hygienic principles, contamination management is not possible. [4, p. 366.] Yeast contaminations can come from process equipment and water, raw materials, food storage and packaging containers. The susceptibility of food to spoilage is affected by chemical, physical and structural properties. Potential yeast contamination may be covered by faster-growing bacteria, which are competing for the same nutrients. The most significant factors for yeast growth in foods are nutrients, acidity, and water activity. More than 70 different contaminant yeast species have been identified and isolated from foods. [21.]

Brewers and other food producers should be aware of the potential hazards of food and regularly monitor critical points in food production; these are required by statutory in-house control. The in-house control system is implemented by the HACCP principles, i.e., the Hazard Analysis and Critical Control Points. The in-house control plan should be appropriately designed for the needs of the operation. It should also include a washing and cleaning plan and a cleanliness monitoring plan, such as surface hygiene monitoring

and testing. [4, p. 366; 22, p. 311-312.] In the brewery, microbiological tests for contaminants, like bacteria and wild yeasts, are made, for example, on air lines, pitching yeast, cooled worts and any materials being added to the beer [17, p. 104]. Cleaning methods and detergents and disinfectants are described in more detail in section 4.3.

Production premises should be located in such a way that the various stages of production can be kept separate, such as raw materials and finished products. Pests such as insects should be prevented from entering production premises. [4, p. 356-357.] Many yeasts travel with the pests, for example, *Lachancea thermotolerans, Saccharomycodes ludwigii* and *Zygotoluraspora florentina* with insects like fruit flies [23]. Premises should be kept clean and tidy so that pests cannot find food there. Insects can be controlled, for example, by placing the lights away above the exterior doors. [4, p. 356-357.] The microbiological quality of the air is particularly important in the filling and packaging departments. Yeasts are most common in the air at soft drink, beer and mineral water bottling lines. [21.] Ventilation should work well to avoid, for example, condensation problems, in which biofilms (Section 4.2) can also form. Working ventilation also promotes the drying of surfaces, for example, after washing. [4, p. 356-357.]

Equipment hygiene has a significant impact on contamination management. Space arrangement and hygienic device designs work best when the device is positioned so it can be easily and thoroughly cleaned. Materials must be selected to endure production conditions such as chemicals, mechanical stress, low and high temperatures, humidity and corrosion. [4, p. 356.] The surfaces of the devices should be smooth and free of protrusions (such as screws), recesses, and sharp edges. The washing water should flow unobstructed from the device. Contaminating biofilms thrive in areas that are difficult to clean and have sufficient nutrients and low water. There are many problematic devices in the food industry, such as tanks, pipelines and filling and packaging machines. [22, p. 315-316.]

Food safety is also greatly influenced by the competence, motivation and hygienic working methods of the staff. Familiarization of staff, maintenance of professional skills and development promote commitment to good operating practices and hygienic working

practices. [4, p. 374] The in-house control plan should have a clear job description, responsibilities and authorizations to be able to determine to whom each job belong. By doing so, it is easier to find the so-called grey areas and repair those. [22, p. 316.]

4.2 Biofilms

There is very little published information on biofilms formed by yeasts, almost all published literature on biofilms in food production deals with bacterial biofilms. Yeast attachment to surfaces and biofilm formation have been studied to some extent in clinical microbiology. Yeast growth is affected by sugar content and type, such as galactose, sucrose and glucose. Yeast growth conditions, such as pH and temperature, affect their attachment. Yeasts have been found to attach to biofilms with bacteria. [21.]

Biofilms are so-called microbial communities. Communities where bacteria, yeasts, mycelial fungi, algae and other micro-organisms can live. Biofilm can in fact be formed wherever it has the necessary nutrients, moisture, micro-organism(s) and a suitable surface to adhere to (Image 1). It is commonly found in moist environments. Many, if not most, biofilms grow in poor nutritional and oligotrophic conditions, such as the depths of the sea and even in the desert. In everyday life, one can see biofilms clogging drains, plaque in teeth, and if you have walked, for example, by the river and slipped on a stone with a thick layer of slime, that slime is biofilm. [24.]

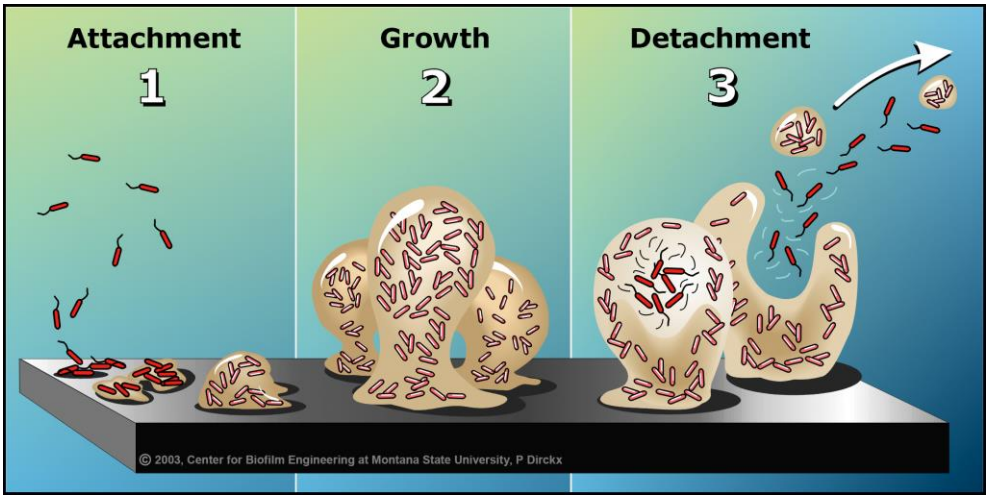


Image 1. Different stages of biofilm formation [24].

Biofilm consists of 85-95% water, the rest are cells, inorganic compounds and polymeric materials which are often fibrous. Biofilm is held by Extracellular Polymeric Substance (EPS). About 50-90% of the biofilm consists of EPS which is produced by cells and enables biofilm to become a complex three-dimensional and flexible adherent community. Biofilms can be as thin as a few layers of cells or many centimeters thick. At higher nutrient levels they are thicker and denser than those growing at low concentrations. EPS is negatively charged and hydrophobic, which allows biofilms to grow under nutrient-free, non-planktonic conditions, as these properties allow the biofilm to concentrate ions and dissolved organic carbon compounds from the oligotrophic bulk liquid.

Getting rid of biofilm can be difficult. Biofilms are dynamic and react to their environment. In addition, microbes can communicate via quorum sensing. Quorum sensing (QS) is essential for biofilm development, if not for getting started. The microbes wait for the group to accumulate before starting action, for example, a marine bacterium that produces light waits for the presence of an adequate population to produce light, or toxin-producing bacteria wait for them to survive the host's defenses. Biofilms can kill other microbes in the vicinity for food and sacrifice microbes living in the community to maintain functional continuity. They can also become dormant if nutrients are depleted or threatened by, for example, antibiotics. Sometimes they are even more resistant to external factors in dormancy. They can also move and divide in both rich and low nutrient conditions. The function of a microbe usually changes considerably when it is transferred to

biofilm. For example, it may take on a new function out of the community and completely change its functions and focus the action on antimicrobial resistance. Biofilms can have up to 1000 times more resistance to antibiotics than planktonic cells. Cells with lower metabolic activity are also less susceptible to the action of antimetabolites such as disinfectants and antibiotics. The biofilm may alter the metabolic activity of the microbes it captures, which can eliminate antimicrobial targets and may increase its resistance. [24; 25.]

If the flow rate is low and there is a laminar flow, it will attract biofilms that do not attach so tightly. Biofilms that occur around high shear rates tend to form long streaks or filaments that lead downstream, are viscoelastic, i.e., they stretch as the flow increases and retract as the flow decreases. They flutter the same way as the flag. Biofilms growing in minor shear environments are usually single microcolonies, which are often irregular and have little or no flow direction. [24.]

Under laboratory conditions, biofilm formation may change. In nature, they have clear functions, but the longer the microbe is handled under laboratory conditions, the more its behaviour is distorted. They may change or cease their characteristic activities. For example, protozoan is not threatening them; thus, they can relax and concentrate on their activities. Life as a single-celled microorganism is not that simple. When these organisms join the biofilm community their complexity will only increase. [24; 25.]

4.3 Cleaning and disinfectants

Maintaining order is essential. Much of the practical work in the brewery, is the cleaning and transporting the wares to the right places. The importance of cleaning in managing contamination cannot be overemphasized. The dirt that accumulates on the surfaces comes from the microbes used, the dust and, above all, the workers. If dirt is visible, it is already too much. All working surfaces should always be cleaned before and after use. [26, p. 56.] Cleaning of equipment and premises is based on chemical and physical methods that are most often used together. Those are cleaned with detergents and disinfectants. Removal of contamination requires mechanical energy, active ingredients and

thermal energy, as well as sufficient time to perform the cleaning. Cleaning methods are selected according to the target and surfaces. The choice is influenced, is it a closed or open process, as well as the size of the target. All washing and disinfection cleaning steps (Table 2) are important. The result is compromised if any step is omitted. Investing in cleaning pays for itself because it guarantees good surface hygiene. [4, p. 366-367.]

Table 2. Washing and disinfection cleaning steps [4, p. 367].

Step	Cleaning implements and execution	Effect
Pre-wash	Cold water and brush or spatula	Removing of proteinaceous loose dirt
Wash or lather	Mechanical wash Application of detergent	Softens and removes dirt
Rinsing	Low pressure	Removing of loose dirt
Drying	Spatula and ventilation	Ensures the required concentration of disinfectant.
Disinfection	Application of disinfectant	Destruction of microbes left on the surface
Rinsing	Low pressure	Removing of residues
Drying	Ventilation	Prevention of potential microbial growth

With basic washing alone, where dirt is removed with detergent, brush or machine, surface rinsing and drying, 90% of the microbes can be removed. Disinfection and basic washing together and adequately performed, destroy more than 99% of microbes. (Table 2) [26, p. 48.]

Detergent and disinfectant effective exposure times can range from a few seconds to days. The effect times depend on the pH, the substance used, the temperature and the target to be destroyed. [26, p. 49.]

When chemically destroying microbes, it must always be remembered that in addition to microbes, the chemical also affects the surface. Therefore, it is important to follow the manufacturer's instructions. Misuse of substances, such as the use of too high a concentration, does not improve efficacy but is likely to leave residues or harmful by-products or end-products in the target. Residues may also be due to poor ventilation or the wrong type of material at the surface. These make it challenging to control surface cleanliness. [26, p 49.]

In breweries, cleaning is essentially similar to other food premises and processes, as stated above (Table 2). Mechanical cleaning in beer production is continuously needed at various sites. Mechanical cleaning by hands is an already familiar form of cleaning that is still suitable for many targets, such as exterior surfaces, corridors, walls, etc. As vessels and tanks became more common in breweries, automation and mechanization of cleaning became necessary. The method that allows the equipment to be cleaned without manual work is called the CIP-method (Clean in Place). In this method, devices are connected to a circulating pump where the cleaning solution circulates. The high flow rate of the circulating solution allows for mechanical cleaning power, especially for cleaning pumps, pipes, valves and heat exchangers. [16, p. 187-188.]

In breweries, caustic soda (usually with sodium hypochlorite) is commonly used as a detergent-sanitizer. The caustic soda dissolves proteins and kills microbes effectively. It is not, however, suitable for keeping in suspension and dissolving calcium salts. Therefore additions of various gluconates, metasilicates or polyphosphates may be made. Some breweries reuse detergent sanitizer because it maintains the level of alkalinity. Alkalinity will fall significantly if carbon dioxide is present. After detergent-sanitizing, a water rinse is necessary and that rinse water can be reused as the first rinse water. The cost of labour is high and therefore many breweries, especially large ones, use automated controls for the washing sequence and recycling. [17, p. 111-112.]

Breweries should have extremely hygienic premises and should be the same level of hygiene as dairies. [17, p. 113.]

4.3.1 Principles of cleaning and disinfection

There are many substances used in chemical cleaning, and they differ in their effectiveness and active ingredients. The most effective and commonly used active ingredients are cationic surfactants and various chlorine compounds and a mixture of hydrogen peroxide and peracetic acid. The most commonly used disinfectants in the industry are disinfectants based on peroxide, alcohol, chlorine and quaternary ammonium compounds. [27.] In detergents and disinfectants, chemical compounds may be microbiostatic or microbicidal, i.e., either stop the microbes in a static state or kill the microbes [26, p. 48].

4.3.1.1 Tensides

Tensides are often used in cleaning agents. Their function is to remove dirt from the surfaces into the water, lower the surface tension of the water and disperse the dirt. That way, it does not adhere to the surface again. The tenside molecule has a hydrophobic and a hydrophilic region. Anionic tensides are used for washing and cleaning and are not microbicidal. These include, for example, natural soap and resin soap. Cationic tensides are quaternary (quat) or tertiary ammonium compounds that are microbicidal. Cationic disinfectants can disperse biofilms to some extent. Quats penetrate the lipid layer of the cell membrane of bacteria and yeast, through their fat solubility and positive charge. Positively charged quats neutralize anionic phospholipids from the membrane, causing the lipid membrane to degrade. [28, p. 33-34.] Quats help to release the biofilm and prevent them from attaching. Quats do not cause taste or odour nuisance or corrosion. [27.]

4.3.1.2 Chlorine compounds

Various chlorine compounds are common ingredients in disinfectants. They are used in premises that require excellent hygiene. [26, p. 52.] Most of them work against microbial cells and viruses [26, p. 37]. Factors affecting the efficiency of chlorine include, for example, temperature, the hardness of the water and the amount of organic dirt, and acidity, which has the most significant effect on its effectiveness. Chlorine compounds are easily inactivated due to dirt and organic material. Chlorine binds electrons, making it a powerful oxidant. The disinfecting properties of chlorine are due to hypochlorous acid molecules with microbicidal properties. [27.]

The oxidant reacts with the purine and pyrimidine bases of DNA as well as cell membranes. Only undissociated hypochlorous acid enters the cell membranes. For this reason, the disinfecting power of chlorine is insufficient at alkaline pH, although the oxidizing power is good. Some microbes are very chlorine resistant and microbes growing as a biofilm tolerate chlorine well. Active chlorine in solution kills free-floating microbes and thus there is less competition for food and biofilms thrive better. [28, p. 38.] Chlorine compounds have long-term effects on materials and, for example, pipelines are stressed by continuous chlorination [26, p. 52].

4.3.1.3 Peroxy compounds

The disinfecting properties of peroxy compounds, i.e., mainly peracetic acid and hydrogen peroxide, are based on their ability to oxidize lipids and proteins. The popularity of their use has increased due to their degradability. [27.] Hydrogen peroxide decomposes into oxygen and water and peracetic acid when diluted with water decomposes into oxygen and acetic acid [26, p. 53]. Hydrogen peroxide is effective against bacteria, yeasts, viruses and spores. It is a clear, water-soluble and pungent-smelling solution. Higher concentrations (10-30%), longer exposure times and/or higher temperatures (50-80 ° C) are required to kill spores, but this can cause corrosion over time. Hydrogen peroxide helps remove dirt, releases oxygen and oxidizes organic dirt. [27.]

Peracetic acid is already effective at 0.3% to spores, viruses, bacteria and fungi. It is a pungent, colorless, clear and oxidizing biocide. Peracetic acid works under both acidic and basic conditions, in the presence of organic dirt, and at low temperatures (2-10 ° C). Its effectiveness is based on the oxidation of sulfur bonds in microbial proteins and enzymes. Peracetic acid effectively removes biofilms, and it is non-foaming, making it also suitable for CIP washes. Concentrated solutions corrode materials. Acetic acid, hydrogen peroxide and water are mixed with peracetic acid. [26, p. 53; 28, p. 36-37; 27.]

4.3.1.4 Alcohols

Alcohols disinfect only in high concentrations. Alcohols are effective against vegetative bacterial cells but are not effective against spores. Alcohol affects cell membrane lipids and proteins. They dissolve lipids from cell membranes, preventing them from continuing to function. [28, p. 45.] Spore-forming bacteria can continue to form spores after alcohol disinfection because alcohol does not denature the enzymes needed to form spores. In chemical disinfection, the good aspects of alcohol are, for example, easy to use, non-toxic, colorless, inexpensive, easily volatile and degradable and do not irritate the skin. [27.]

4.4 Preservatives

Preservatives are substances that protect food from spoilage by microorganisms [29]. Bacteria, yeasts and moulds spoil foods that are stored longer. The spoilage process is slow, especially if the product is stored cold. Preservatives can be added to products to improve shelf-life, and they reduce the growth and development of microorganisms. Microorganisms can cause, for example, fermentation, putrefaction and mould formation. Chemical preservation reduces the risk of the formation of toxic substances that moulds and bacteria can produce. The nutrient content and taste of foods can be better preserved due to preservatives. The use of preservatives in some foods can be reduced by cold storage, freezing as well as a packaging technology. [30, p. 25.]

4.4.1 Naturally occurring growth inhibition in different drinks

Natural factors are composition, pH, buffering capacity, oxidation-reduction potential (redox) and water activity (a_w). These are part of the food itself. [31, p. 338.]

For most microbes, beer is a poor breeding ground. Low pH (3.8-4.7) and ethanol inhibits their growth well. Beer also has a limited number of carbon sources for microbial nutrition. Hops inhibit the growth of gram-positive bacteria, and the low oxygen content inhibits the growth of aerophilic microbes. The beer itself does not support growth of pathogenic bacteria, but mould toxins that enter the beer with the raw materials or are created during production pose a risk to the consumer. [4, p. 239-240.]

Soft drinks and juices are naturally rich in organic acids from berries and fruits, such as citric, tartaric and malic acids. Cola drinks also contain phosphoric acid. The natural pH value of these drinks is 2.0-4-5. In juice concentrates, the water activity can be slight, and the sugar contents up to 40-60%. The low redox potential inhibits the microbial growth of beverages. In pasteurized products, the removal of oxygen and the formation of sulfites from the fruit particles by ascorbic acid also reduce the redox potential. Many berries and fruits contain antimicrobial agents naturally, such as oils, benzoic acid (e.g., cranberries and lingonberries), and sorbic acid (e.g., rowanberries). [4, p. 260-261; 30, p. 26]

The concentration of a single substance does not guarantee preservation, but they complement each other and support other preservation methods [4, p. 261].

4.4.2 Preservatives against yeasts and their functional mechanisms

It is not always possible to eliminate microbes by physical methods. Therefore, antimicrobial agents, like preservatives, are needed. Using weak acids as preservatives, their

pKa value and pH value of the food are essential for the application. Only the undissociated molecule can penetrate the inside of the microbial cell. Therefore, for acidic foods, weak acids are more suitable. [32, p. 456.]

4.4.2.1 Benzoic acid

Benzoic acid is common in nature (e.g., in plum, bilberry and cranberry) as a glycoside. Its activity is directed both to cell walls and inhibition of citrate cycle enzymes and enzymes involved in oxidative phosphorylation. Benzoic acid and its salts are more effective against yeasts and moulds than bacteria. Benzoic acid is often combined with other preservatives. It has higher activity in acidic foods (pH 4-4.5 or lower). [32, p. 456; 30, p. 26.]

4.4.2.2 Sorbic acid

Sorbic acid occurs naturally, for example, in rowan berries. Sorbic acid and its salts are more effective against yeasts and fungi than bacteria. The activity depends on pH, and its utilization is up to pH 6.5. The advantage of sorbic acid is that it is odorless and tasteless at the level of use. Biochemically, sorbic acid degrades like a fatty acid. [32, p. 458; 30, p. 26.]

4.4.2.3 Sulfur dioxide

Sulfur dioxide and sulfite are effective against yeasts, moulds and bacteria. The activity increases with decreasing pH. Mostly because of undissociated sulfurous acid, which predominates at a pH < 3. Sulfite reacts with several food constituents, for example, with various cofactors, for example, folic acid, pyridoxal and thiamine and with proteins with cleavage of disulfide bonds. Sulfur dioxide is not only antimicrobially active but also prevents discoloration by inhibiting compounds with a reactive carbonyl group or by preventing the oxidation of phenols by phenol oxidase enzymes. [32, p. 459; 30, p. 27.]

4.4.3 Preservatives and legislation related to their use

Almost all preservatives have either food-specific or quantitative use restrictions [30, p. 25].

Food and Agriculture Organization (FAO) and the World Health Organization (WHO) Expert Committee on Food Additives (JECFA) evaluate the safety of additives internationally. The European Food Safety Authority (EFSA) assesses the safety of food additives in Europe. [30, p. 10.] The possible adverse effects of the preservatives are considered in the report. On the basis of the safety report, the acceptable daily intake of the substance is determined. If no adverse effects occur even in large quantities, no ADI (Acceptable Daily Intake) value is set for the preservative. ADI means the maximum acceptable daily intake that a person can be exposed to each day without adverse effects. [33.] For example, the maximum amount of benzoic acid in juice is 200 mg / l, and the ADI is 5 mg/kg/d; thus, a person weighing 60 kg could drink 1.5 l of juice a day without exceeding the ADI. [30, p. 10.]

4.4.3.1 Benzoic acid

The maximum level of benzoic acid for juices and non-alcoholic keg beer and alcoholic beverages with an alcoholic strength of less than 15% vol, is 200 mg/l. For non-alcoholic beverages it can be added up to 150 mg/l. The ADI value for benzoic acid is 5 mg/kg/d. [29; 30, p. 26.]

4.4.3.2 Sorbic acid

The maximum level of sorbic acid for non-alcoholic keg beer, alcoholic beverages with an alcoholic strength of less than 15% vol, wine-based aromatized drinks (including non-alcoholic) and mead is 200 mg/l. Sorbic acid can be added to juice by 500 mg/l and non-alcoholic flavored drinks by 300 mg/l. The ADI value for sorbic acid is 25 mg/kg/d. [29; 30, p. 26.]

4.4.3.3 Sulfur dioxide

The maximum level of sulfur dioxide for beer (including NABLAB) and non-alcoholic beverages that contain fruit juice are 20 mg/l. Sulfur dioxide can be added to ciders and fruit wines (including sparkling wines) by 200 mg/l. The ADI value for sulfur dioxide is 0.7 mg/l. [29; 30, p. 27.]

4.5 Temperature

The most important microbial environmental condition is temperature [26, p. 110]. All microbes have their characteristic growth temperature range. Temperature ranges for yeasts are from 2 °C to about 47 °C. [34, p. 299.] Optimal temperatures for growth are between 15 °C to 35 °C, but most yeasts grow best at 20 °C to 25 °C [35, p. 88]. When the temperature is close to the upper limit of tolerance, it consumes more energy. For example, enzymes denature at sufficiently high temperatures, and their lifespan is shortened; therefore, the cell has to change them more often. [28, p. 248.] If the maximum temperature is maintained long enough, the microbe will eventually die. As the temperature drops to the freezing temperatures, microbial metabolism slows down and they no longer divide [26, p. 110-111.] Yeasts that are in vegetative form can be killed in 10-20 minutes by heat treatment at 55 °C to 60 °C. Increasing temperature up to 70 °C yeast ascospores can be destroyed [31, p. 373].

4.5.1 Pasteurization, pros and cons

The goal of pasteurization is to reduce the total number of microbes and to destroy disease-causing microbes. However, pasteurization does not affect spore forms or viruses, therefore, it cannot completely destroy microbes. The method is particularly suitable for liquid foods such as beer, juice and milk. Pasteurization is widely used in the food and beverage industry. It improves the shelf life of the product and is, therefore, safer to use. [26, p. 41.]

Pasteurization means the destruction of microbes using heat (below 100 °C) [36, p. 44]. Pasteurization temperature and processing times vary by product. For example, when pasteurizing milk, the temperature is raised to 72 °C for 15 seconds, and the typical heat treatment of beer is 30 seconds at 72 °C. [26, p. 41; 22, p. 189]

Two different methods are used to pasteurize beer; tunnel pasteurization and plate pasteurization. Plate pasteurization is a more used method. Tunnel pasteurization is expensive, and the flavor of the beer deteriorates. In tunnel pasteurization, filled and closed cans or bottles are passed through a tunnel set to the pasteurization temperature. This method is commonly used in exported beers because it guarantees biological preservation. In plate pasteurization, the beer is passed from the filter through a heat exchanger. [16, p. 164-166.]

The pasteurization of beer affects the taste and colour of the beer. In bottles and cans, pasteurization easily gives the beer a darkening of the colour and an oxidized flavour (bread-like). The intensity of the changes depends on the amount of oxygen in the cans and bottles. In tunnel pasteurization, the amount of oxygen in the package must be minimal. [16, p. 165-166.]

4.5.2 Fridge temperatures

Microbiological contamination is slow, especially when it occurs in the cold [36, p. 43]. Cold/cool storage occurs between 16 to -2 degrees. As the temperature decreases, the chemical reactions decrease, which is directly related to the growth rate of the microbes. The refrigerator temperature is lower than the minimum growth temperature of many food-contaminating microbes. The growth of cold-tolerant psychrotolerant microbes also slows down at low temperatures. However, if the product already has a large population of microbes, the food can spoil quickly even when stored in the refrigerator. [31, p.364-365.]

Beer abides best in the dark and cold. Properly stored, beer can be stored for an acceptable sales period, which in Finland is usually six months. [22, p. 187.] The uninterrupted cold chain is an absolute prerequisite for the preservation of foods, such as beer. During transport, even a short-term rise in temperature can decisively increase the number of microbes. When private consumer transports products, temperatures can rise to high levels, for example, in summer. Thus, it would be good for the consumer to be aware of the disadvantages of foods when transported and stored warm. Also, restaurants and bars that do not have cold storage rooms should observe the temperature. [36, p. 47.]

5 Materials and methods

5.1 Yeast strains

Twenty-five different yeasts were used in this thesis project (Table 3).

Table 3. All used yeasts in this thesis with their codes.

Genus	***Species***	***Code***
Babjeviella	*inositovara*	100-1
Brettanomyces	*anomalus*	C-75001T
	bruxellensis	C-05796
	bruxellensis	WLP650
Candida	*zemplinina*	C-181019
Cyberlindnera	*fabianii*	
Hanseniaspora	*uvarum*	FA4
Kazachstania	*servazzi*	
Kluyveromyces	*marxianus*	Km2
Lanchea	*fermentati*	C-09854T
	thermotolerans	C-16989
Metschnikowia	*sinensis*	FA7
Mrakia	*gelida*	4GW184
Pichia	*fermentans*	

	kluyveri	C-00354
Saccharomyces	*cerevisiae*	WLP380
	cerevisiae var. diastaticus	Belle Saison
	cerevisiae var. diastaticus	C-70060
	pastorianus	W34/70
	paradoxus	C-16968
Saccharomycodes	*ludwigii*	
Torulaspora	*delbrueckii*	8bA13
Wickerhamomyces	*anomalus*	C-74021T
Zygosaccharomyces	*rouxii*	C-94197T
Zygotorulaspora	*florentina*	OTA

Most of the yeasts are known contaminants in the food and beverage industry. Some of the yeasts are relatively poorly known, such as *K. servazzi*, which has occurred as a contaminant at VTT's pilot brewery.

5.2 Biofilm-forming potential

Yeast biofilm production was tested by microplate culture with 96 wells. Yeast were divided into two groups. Strains were propagated by taking a loopful of fresh yeast mass from YPD agar and inoculating into 25 ml of liquid YPD and incubating for one day on a shaker (120rpm) at room temperature. Grown cell cultures' optical densities (OD_{600}) were measured with a spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan). On the basis of the lowest optical density, the yeasts were diluted for growth. Eighty-four wells were filled with 250 µl 10 °P inoculation wort, including 65 well for yeasts and 19 for blank samples. The rest of the wells were empty or filled with water. 2.5 µl of culture was used to inoculate five replicate wells. Growing times were 24 h or five days, without disturbance at room temperature. After 24 h or five days OD_{600} vales were measured from each yeast from one well, to be sure there has been growth. Lager yeast *S. pastorianus* (W34/70) used as a reference in every growth.

Biofilm-forming potential was assessed by measuring attachment of the cells to the walls of the wells. The plate was first rinsed with sterile Milli-Q-filtered water. After which, 300

µl of 0.1 % crystal violet solution was placed in the wells for five minutes. It was then rinsed three times with sterile mQ water. The plate was left to air-dry for 15 minutes in a laminar flow cabinet. The remaining crystal violet, which was still bound to the cells, was dissolved with 300 µl of 95 % Etax B (AaS, Rajamäki, Finland) (Image 2). Absorbance of the wells were measured at 595 nm with Multiskan EX (Labsystems Oy, Finland). The plate was left overnight at fridge temperature and absorbance re-measured.

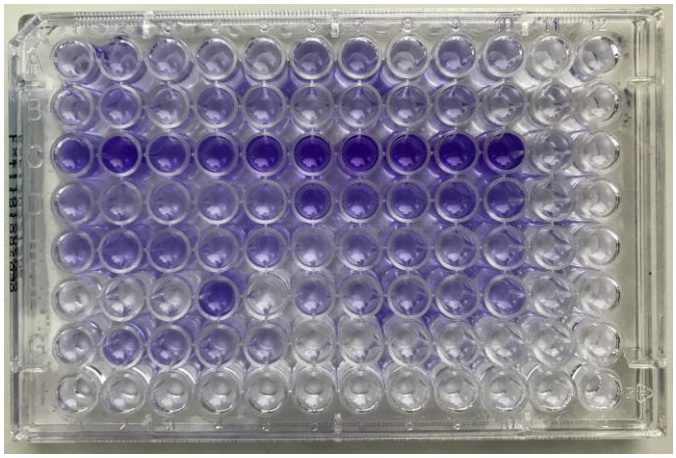


Image 2. Growths of the biofilm-forming potential. The remaining crystal violet, which was still bound to the cells, was dissolved by 95 % Etax B.

Means were calculated using measurements of five strain against the mean of measurements of five blank samples (Microsoft Excel, t-test). In this way, it can be analyzed if the growth was statistically significant.

5.3 Disinfectant resistance

The tolerance of yeasts to the disinfectant 3 % P3-oxonia active (Oy Ecolab Ab, Helsinki, Finland) was tested as follows. YPD agar plates were prepared with supplements of serum albumin (BSA) (Sigma-Aldrich, Missouri, United States). One concentration of BSA represented ‘clean’ conditions (0.3 % BSA), and one represented ‘unclean’ condition (3

% BSA). BSA dilutions were made with sterile mQ water. Yeast suspensions were prepared by taking a loopful of fresh yeast mass from a YPD agar plate culture and inoculating into 5 ml of sterile physiological saline solution (0.9 % NaCl).

Yeast solution was added at 60 µl for both conditions, to 3 ml of 0.3 % BSA and to 3 ml of 3 % BSA. Each yeast had two agar plates (divided by 5 sample times). Before adding disinfectant, both solutions with yeast was added to agar plates by spread plate for controls. The disinfectant was added at 300 µl for both conditions, and timing started after adding. Times were 2.5, 5, 10 and 20 minutes. In each timing, solutions were mixed well by Vortex® and then added a loopful from solutions to agar plate by spread plate. The plates were incubated at 25 °C, except *M. gelida* at 10 °C, for three to five days, depending on the growth yield.

Resistance to the oxonia was assessed qualitatively depending on the growth of the yeasts.

5.4 Preservative tolerance

The tolerance of the yeasts to common food preservatives was assessed in microplate cultivations (Image 3) using a Bioscreen C incubator and plate reader (Labsystems Oy, Finland). Yeasts were propagated by taking a loopful of fresh yeast mass from YPD agar and inoculating into 25 ml of liquid YPD for one day on a shaker (120rpm) at room temperature. The culture was then centrifuged at 4000 rpm for 5 min at 1 °C. The pellet was washed with 10 ml of sterile 0.9% NaCl. A 20%-slurry were prepared, and cell density was measured with NucleoCounter YC-100 (Chemometec, Denmark). The microplate's wells were filled with 150 µl of YPD (1% glucose w/v) and with 140 µl of one of the following preservatives isomerized hop extract IsoHop® 30 IBU (BarthHaas, Nuremberg, Germany), ethanol 5% (v/v) (AaS, Rajamäki, Finland), benzoate 150 mg/l (sodium benzoate, Sigma-Aldrich, Darmstadt, Germany), sorbate 250 mg/l (potassium sorbate, Sigma-Aldrich, Darmstadt, Germany), and sulfite 200 mg/l (potassium metabisulfite, Brown, Poland). All media were adjusted to pH 4 with HCl. Yeast was added at 10 µl per well to obtain approximately 2000 cells per well. The final volume was 300 µl per well,

and the cultivations were carried out at 20-25 °C with moderate shaking. Each culture was prepared in triplicate for each preservative. Lager yeast *S. pastorianus* was used as a reference in every run.

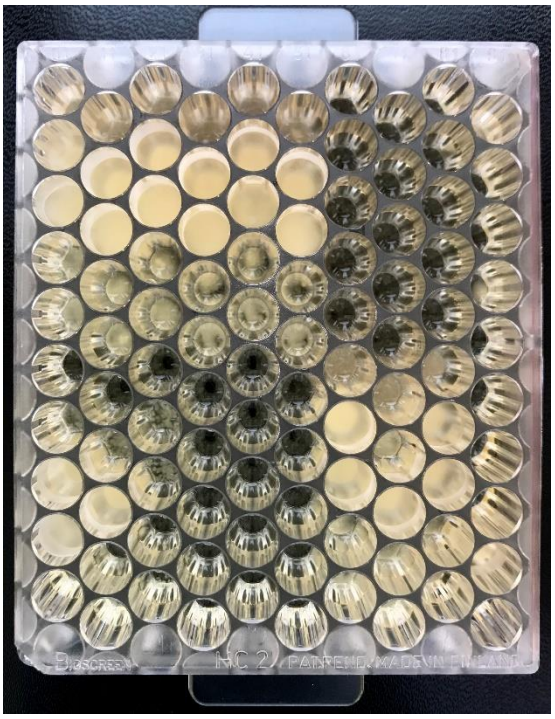


Image 3. Microplate cultivations after growth with media and yeast.

Means were calculated with Microsoft Excel. From the results, growth rate, maximum growth, lag phase were calculated, and graphs were drawn to illustrate the growth.

5.5 Temperature

The ability of the yeasts to grow at different temperatures was tested using a spot plate technique. Yeasts were propagated by taking a loopful of fresh yeast mass from YPD agar and inoculating into 25 ml of liquid YPD for one day on a shaker (120rpm) at room temperature. Each culture was centrifuged, washed and re-suspended to OD_{600} of 0.1 (2×10^6 cells per ml) and further diluted to concentrations of 0.01, 0.001 and 0.0001 using sterile Milli-Q-filtered water. 5 µl of each suspension was spotted onto the surface

of a YPD agar plate. The plates were incubated at 37 °C for three days, 4 °C for two weeks and 1 °C for three weeks. The lager yeast *S. pastorianus* (W34/70) was used as a reference in every plate.

6 Results and discussion

6.1 Ability to form biofilm

This experiment aimed to determine the propensity of different wild yeasts to produce biofilm. The microbes need to be able to attach to a surface to form it. As microbes combine with biofilm, their tolerance to external stressors such as cleaning increases. Their resistance to, for example, disinfectants can also increase by as much as a hundred-fold when they adhere. Biofilms can be a significant problem with respect to process hygiene. Despite cleaning, they may remain under challenging areas such as corners and furrows. Dirt on surfaces, such as grease and proteins, usually promotes activity. If the conditions remain favorable for the biofilm and it is allowed to remain a long time, the more difficult it is to remove. For this reason, the resulting biofilm and adherent cells must be removed during cleaning daily. [4, p. 370.] Biofilm can also divide and move [24]. This experiment was done in two batches. Growing times were between one and five days at room temperature.

Most of the yeasts produced biofilm. Only five of the 25 yeasts did not produce it. These included *B. inositovara*, *C. zemplinina, M. gelida*, *P. fermantans*, and *Z. rouxii*. However, this does not preclude their production of biofilms. Sometimes yeasts biofilm formation can depend on the day. For example, *C. zemplinina* is often isolated from wines, grape juices and from barrels which are contaminated with biofilm communities. It often occurs with other *Candida* species in biofilms. The producing of *C. zemplinina* and *M. gelida* biofilm can be very different when exposed to colder conditions because both are psychrophilic. [37; 38, p. 1508.]

Six yeasts behaved differently compared to other yeasts. They produced a statistically significant amount of biofilm over one day, but in the five-day culture, the yield was either non-existent or very low. This behavior can be explained, for example, by depletion of nutrients for these yeasts and which may have been entirely or only partially detached during rinsing and staining. These included *B. bruxellensis* (WLP650), *H. uvarum*, *K. marxianus*, *L. fermentati, M. sinensis*, and *W. anomalus*. Eight yeasts did not produce or produced very little biofilm during the day, but after the fifth day, the yield was statistically significant. This behavior is very typical for many microbes, and adequate time is an important part of biofilm formation. These yeasts were *B. anomalus, S. cerevisiae, S. cerevisiae var. diastaticus* (Belle Saison), *S. cerevisiae var. diastaticus* (C-70060), *S. pastorianus*, *S. ludwigii*, *T. delbrueckii* and *Z. florentina*. The remaining six yeasts produced a statistically significant amount of biofilm during both one and five days. These included *B. bruxellensis* (C-05796), *C. fabianii, K. servazzi, L. thermotolerans, P. kluyveri*, and *S. paradoxus*.

On the basis of the experiment, most of the studied yeasts are potential biofilm producers. It is very usual in biofilms to have a variety of microbes. As different microbes combine with biofilm, it can be even more challenging to get rid of them. That is, if one of these yeasts produces biofilm in production, other microbes may combine there and thus increase diversity as well as the need for cleaning. [24.]

6.2 Disinfectant resistance

The disinfectant test was intended to test how yeasts can grow without the protection provided by the biofilm, under both unclean and clean conditions, after use of a disinfectant. It is necessary to know how a disinfectant affects cells if adequate hygiene is to be maintained. Thus, the need for purification of the yeast-produced biofilm can also be partially assessed. The disinfectant ensures the cleanliness of the spot. They are used only in the final stages of cleaning. Disinfectants are not a substitute for other steps and do not diminish the importance of other steps, such as mechanical washing. [4, p. 366-367.] 0.3% BSA represented the clean condition in the experiment, and 3% BSA represented unclean conditions, where disinfectant applied may react with dirt and grease on

the surface and have less effect on microorganism. P3-oxonia active was selected as the disinfectant. It is a common disinfectant in the food industry for disinfecting pipelines, equipment and tanks. It is effective against bacteria, fungi, viruses and spores. [27; 39.] VTT's Department of Bioprocess Technology also uses P3-oxonia active; thus, it was easily available. In this experiment exposure lasted for 2.5, 5, 10 or 20 minutes. There was also a control with no disinfectant.

Most yeasts did not grow under clean conditions after disinfection. This group included, for example, *B. bruxellensis* (WLP650), *L. thermotolerans*, *P. kluyveri*, and *S. pastorianus*. (Table 4) Under this condition, only *B. bruxellensis* (C-05796) and *K. servazzi* grew slightly even after 20 minutes (Image 4). *B. bruxellensis* is a familiar contaminant, for example, in the production of soft drinks, wine, cider and beer [23, p. 376]. For 2.5 minutes, survived both *S. cerevisiae var. diastaticus* (Belle Saison & C-70060) and *T. delbrueckii* (Table 4).

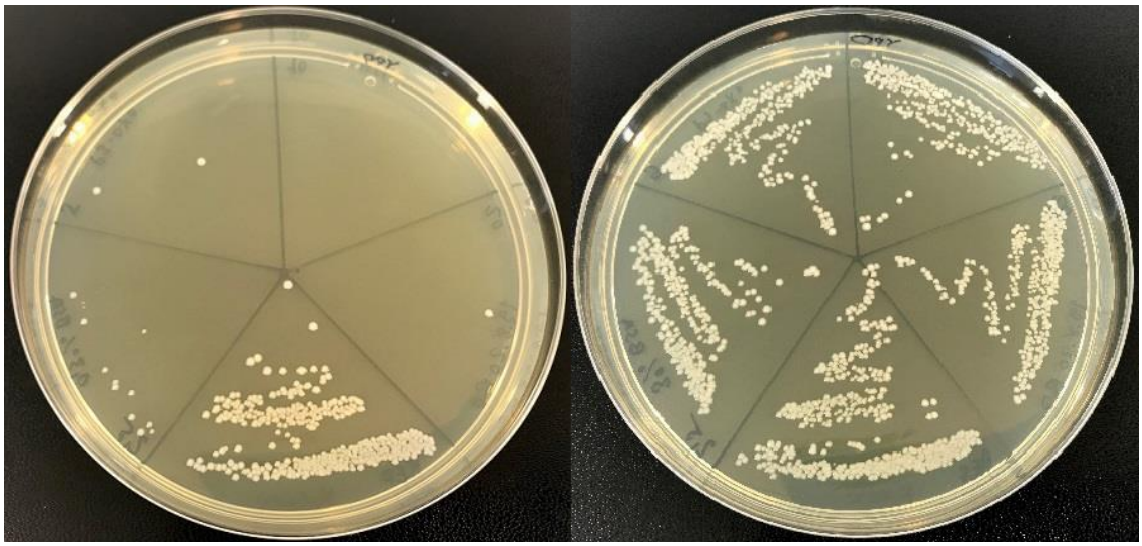


Image 4. *K. servazzi* growth at clean conditions at 0.3 % BSA (left) and unclean conditions at 3% BSA (right).

In unclean conditions, most yeasts succeed better. Only *L. fermentati* and *M. gelida* did not grow after disinfection. Ten yeasts survived in unclean conditions even after 20 minutes of exposure. Five of these did exceptionally well compared to the others. These

were *B. bruxellensis* (C-05796), *K. servazzi*, both *S. cerevisiae var. diastaticus* (Belle Saison & C-70060) and *T. delbrueckii*. These five also produced biofilm well, thus, they can cause a long-term inconvenience if they get into production. In a biofilm, the microbial tolerance to stressors multiplies. [24.]

Table 4. Estimates of yeasts growths after disinfection at different times. +: positive -: negative w: weak.

Yeast	Control growth, 0,3 % BSA	2,5 min, 0,3 % BSA	5 min, 0,3 % BSA	10 min, 0,3 % BSA	20 min, 0,3 % BSA	Control growth, 3% BSA	2,5 min, 3 % BSA	5 min, 3 % BSA	10 min, 3 % BSA	20 min, 3 % BSA
B. anomalus	+	-	-	-	-	+	w/-	-	-	-
***B. bruxellensis* (C-05796)**	+	+/w	w	w	w	+++	+	++	++	++
***B. bruxellensis* (WLP650)**	+	-	-	-	-	+	+	+/w	+/w	+
C. zemplinina	w	-	-	-	-	w	w/-	-	w/-	-
K. servazzi	++	+/w	w	-	w/-	+++	++	++	++	++
L. fermentati	+	-	-	-	-	++	-	-	-	-
L. thermotolerans	++	-	-	-	-	+	+	+/w	w	w/-
M. sinensis	++	-	-	-	-	-	w	w/-	w/-	-
M. gelida	++	-	-	-	-	+	-	-	-	-
P. fermentans	+	-	-	-	-	+	w	w/-	-	-
P. kluyveri	+	-	-	-	-	+	w	-	-	-
***S. cerevisiae var. diastaticus* (Belle Saison)**	++	w/-	-	-	-	++	++	++	++	+
***S. cerevisiae var. diastaticus* (C-70060)**	+++	+	-	-	-	++	++	++	++	++
S. paradoxus	+	-	-	-	-	+	+	+/w	w	w/-
S. pastorianus	+	-	-	-	-	+	-	w/-	-	-
S. ludwigii	w/-	-	-	-	-	w/-	w/-	w/-	w/-	w/-

T. delbrueckii	+++	++	-	-	-	++	++	++	++	+
Z. rouxii	+	-	-	-	-	+	+	+/w	+/w	+/w
Z. florentina	++	-	-	-	-	+	w	w/-	w/-	-

On the basis of the results, in case of possible contamination, it would be good to have the yeasts destroyed while still in the cellular state. It is also good to note that there was only one disinfectant in the experiment. Different disinfectants have different modes of action. Disinfectants, for example, should be alternated between different substances. This way it is also possible to avoid the development of microbial resistance to disinfectants. Contamination management is based on the cleaning of equipment and premises and prevention. Without adequate knowledge of hygienic principles, contamination management is not possible. [4, p. 366-367.] *B. inositovara, C. fabianii, H. uvarum, K. marxianus* and *W. anomalus* did not grow in the experiment at all. It may be that they were no longer alive on the YPD dish from which they were inoculated.

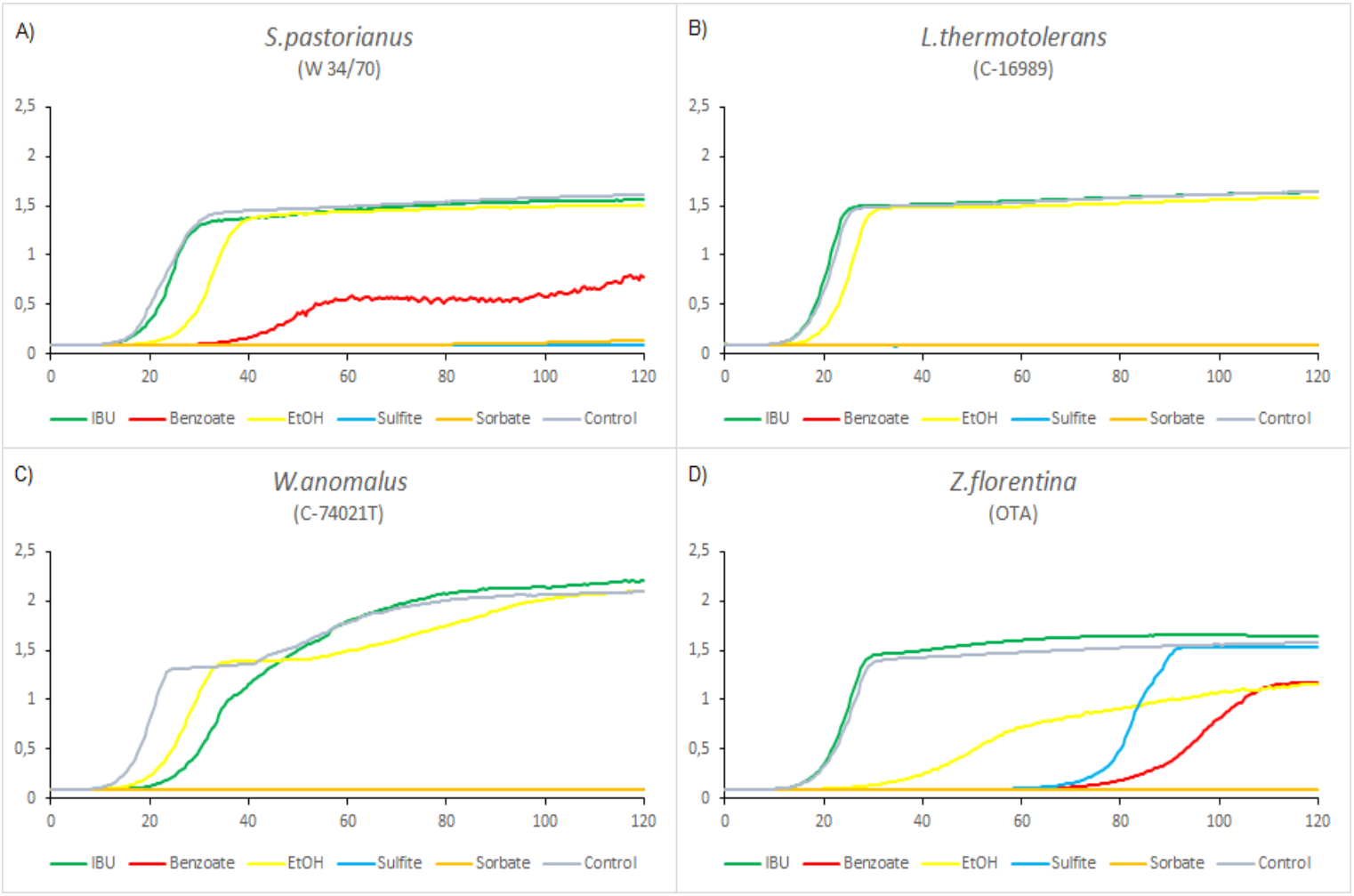
6.3 Preservative tolerance

This experiment focused on yeast strains' ability to tolerate different preservatives. Preservatives prolong the shelf life of foods by protecting them from contamination by micro-organisms as well as from the growth of pathogenic micro-organisms [40]. It is important to know which preservatives work best for alternative brewing yeasts. Especially on how they inhibit the growth after contamination. Most of the yeasts that are studied in this thesis are known contaminants in the food and beverage industry, for example, *P. fermentans* and *W. anomalus*. Also, when looking for different yeasts for known uses, such as brewing, it is important to know how the yeast behaves under different conditions. By using preservatives, the survival of many yeasts and the continuation of activity in the product can be excluded. Especially when no other preservation methods, such as pasteurization, are used. Preservatives used were IsoHop (30 IBU), ethanol (5 %), benzoate (150 mg/l), sorbate (250 mg/l) and sulfite (200mg/l). These preservatives are commonly used in food, soft drinks, alcohol and non-alcoholic beverages. The acceptable daily intake (ADI) of these preservatives is 5mg/kg/d for benzoate, 25mg/kg/d for sorbate and

0,7mg/kg/d for sulfite. [30, p. 26-27.] All preservatives had some effect on yeast as shown below.

IsoHop (IBU) i.e., iso-α-acid has an antimicrobial effect and it adds a more bitter flavour to the beer [41]. IsoHop had no significant effect on the yeasts and it did not inhibit any yeast's growth completely. 12 yeasts from a total of 25 strains, for example, *B. anomalus, B. bruxellensis* (WLP650) and *K. servazzi* had similar or higher growth rates with IsoHop than the control (Appendix 1; 2, Table 5). For 9 yeasts, including *H. uvarum*, *L. thermotolerans* and *P. fermentans*, the maximum growth values were the same or higher with IsoHop exposure compared to the maximum control value of the control (Appendix 1; 2, Figure 1B). Most of the yeasts grew to levels similar to that of the control. IsoHop delayed the growth of *W. anomalus* most; it started to grow after 9 hours, when the control started to grow after only 4.5 hours (Table 5, Figure 1C). Compared to the lag phase of the control, that of *B. inositovara* lag phase with IsoHop was lowest, it started to grow 11.5 hours before control (Appendix 1; 2).

Figure 1. Growth curves with IsoHop (IBU), benzoate, ethanol, sulfite, sorbate and control for *S. pastorianus* (A), *L. thermotolerans* (B), *W. anomalus* (C) and *Z. florentina* (D). The x-axis indicates time, and the y-axis shows the yeast growth.



Ethanol did not have a major effect on the yeast's growth, it mainly extended the lag phase. For example, it delayed the initiation of growth of *S. cerevisiae* until 23.5 hours, while, for example, *C. fabianii, P. fermentans* and *W. anomalus* started to grow 0.5 hours after the control (Table 5, Appendix 1; 2). Only *B. anomalus*, *K. servazzi*, *M. sinensis*, *S. cerevisiae* and *Z. florentina* growth were clearly inhibited by ethanol (Table 5, Appendix 1; 2). *B. inositovara* and *M. gelida* growth was inhibited completely by ethanol.

Benzoate is effective in acidic foods against yeasts, mold and certain bacteria [30, p .26]. Benzoate was the most inconsistent from all preservatives. Its effects on yeasts were

very variable. With the reference yeast, *S. pastorianus*, the growth kinetics varied, but at least results were consistent (Figure 1A). *B. inositovara*, *H. uvarum*, *L. thermotolerans*, *M. sinensis*, *M. gelida* and *W. anomalus* growth was completely inhibited by benzoate. On average it delayed the growth about by 24 hours. From all the yeasts, benzoate delayed *S. cerevisiae* growth the most, by 65 hours. Apart from the delay, benzoate did not have much effect on *S. cerevisiae* growth (Table 5). Compared to the control, all the yeasts that grow with benzoate, benzoate decreased their growth rates by about 52 % and maximum growths by about 53 %. *B. anomalus*, *L. fermentati*, *S. paradoxus* and *S. ludwigii* were not significantly affect by benzoate. On average, their maximum growths were decreased by only 38 %. Benzoate decreased *B. bruxellensis* (C-05796), *B. bruxellensis* (WLP650), *C. fabianii*, *P. kluyveri* and *S. pastorianus* growth rate the most, about 75 %.

Sorbate is an effective preservative especially in acidic and slightly acidic foods against yeasts, mold and certain bacteria [30, p .26]. Sorbate delayed and decreased the growth of many yeasts. For 17 yeast from a total of 25 yeasts, growth was completely inhibited by sorbate. This, for example, was the case with *T. delbrueckii*, *Z. rouxii* and *Z.florentina* (Appendix 1; 2). Yeasts that grew with sorbate, for example, *S. pastorianus*, *P. kluyveri* and *S. ludwigii* growth were delayed on approximately by 38 hours (Table 5). *B. anomalus* started to grow fastest with sorbate. This preservative delayed *B. anomalus* growth for only 23 hours (Appendix 1; 2). Compared to the growth of the control, on average, sorbate decreased the growth rate by about 84 % and the maximum growth by 86 %. *B. bruxellensis* (WLP650) was a clear exception, sorbate decreased its growth rate by only about 38 % and its maximum growth was decreased by 50 %.

Sulfite is a traditional preservative that is especially effective against bacteria. It also has an antioxidant effect. [30, p .27] Sulfite was effective at inhibiting growth. Growth of 21 yeasts was completely inhibited by sulfite. Sulfite had no significant effect if the yeast were growing, but at least it delayed growths quite well. *Z. rouxii* was not affected significantly by sulfite. Its growth rate and maximum growth were the same as control. Sulfite only delayed *Z. rouxii* growth by 26 hours. (Appendix 1; 2) Sulfite was most effective against *L. fermentati*. Its growth was delayed for 34.5 hours. Compred to the growth of

the control, the growth rate and maximum growth were decreased by 35 % and 32 % by sulfite (Appendix 1; 2). *S. ludwigii* is known to survive even high amounts of sulfite, ones that most of the yeasts would not survive. It is a common contaminant yeast in productions with fermented beverages that use sulfite as a preservative, such as cider and wine. [23, p. 749.] The growth of *S. ludwigii* was delayed for 20 hours by sulfite. Compared to the control, its growth rate decreased by approx 14 % and maximum growth by 35 % (Table 5). Sulfite delayed *Z. florentina* growth for 46 hours and decreased growth rate by 10 % and maximum growth by about 18 %. (Appendix 1; 2, Figure 1D) Yeasts that grew with sulfite are common contaminants in beverages such as fruit juices, soft drinks and wine [23].

Table 5. Different conditions (control, IsoHop, benzoate, ethanol, sulfite and sorbate) and growths from *S. pastorianus*, *K. servazzi, K. marxianus, P. kluyveri, S. cerevisiae, S. ludwigii* and *W. anomalus*

Yeast	*S. pastorianus*	*K. servazzi*	*K. marxianus*	*P. kluyveri*	*S. cerevisiae*	*S. ludwigii*	*W. anomalus*
Control lag phase (h)	5 ± 0,04	3,5 ± 0,02	3 ± 0,04	3 ± 0,05	5 ± 0,14	4 ± 0,05	4,5 ± 0,04
Control growth rate	0,22 ± 0,07	0,26 ± 0,02	0,28 ± 0,04	0,25 ± 0,15	0,25 ± 0,14	0,22 ± 0,06	0,22 ± 0,02
Control maximum growth	0,11 ± 0,01	0,34 ± 0,005	0,22 ± 0,005	0,19 ± 0,006	0,14 ± 0,008	0,2 ± 0,008	0,17 ± 0,007
IsoHop lag phase (h)	4,5 ± 0,074	6,5 ± 0,04	5 ± 0,04	3,5 ± 0,02	7 ± 0,17	3,5 ± 0,03	9 ± 0,09
IsoHop growth rate	0,23 ± 0,1	0,3 ± 0,04	0,28 ± 0,03	0,25 ± 0,12	0,29 ± 0,2	0,2 ± 0,05	0,15 ± 0,07
IsoHop maximum growth	0,18 ± 0,01	0,22 ± 0,006	0,21 ± 0,004	0,16 ± 0,006	0,13 ± 0,008	0,14 ± 0,06	0,11 ± 0,01
Ethanol lag phase (h)	10 ± 0,06	20 ± 0,18	5,5 ± 0,02	6 ± 0,02	23,5 ± 0,15	6,5 ± 0,04	5 ± 0,04
Ethanol growth rate	0,18 ± 0,06	0,08 ± 0,3	0,22 ± 0,02	0,2 ± 0,02	0,19 ± 0,07	0,2 ± 0,07	0,17 ± 0,04
Ethanol maximum growth	0,14 ± 0,005	0,07 ± 0,02	0,18 ± 0,004	0,14 ± 0,005	0,05 ± 0,004	0,14 ± 0,006	0,12 ± 0,007
Benzoate lag phase (h)	19,5 ± 0,21	21 ± 0,17	25 ± 0,02	10,5 ± 0,05	65 ± 0,04	7 ± 0,05	N/A
Benzoate growth rate	0,07 ± 0,6	0,13 ± 0,3	0,08 ± 0,8	0,15 ± 0,1	0,16 ± 0,09	0,18 ± 0,08	N/A
Benzoate maximum growth	0,12 ± 0,04	0,11 ± 0,02	0,05 ± 0,003	0,08 ± 0,008	0,1 ± 0,006	0,14 ± 0,008	N/A
Sorbate lag phase (h)	40 ± 0,003	N/A	N/A	68 ± 0,005	N/A	34 ± 0,013	N/A
Sorbate growth rate	0,012 ± 0,03	N/A	N/A	0,006 ± 0,07	N/A	0,03 ± 0,09	N/A
Sorbate maximum growth	0,003 ± 0,0006	N/A	N/A	0,001 ± 0,001	N/A	0,025 ± 0,003	N/A
Sulfite lag phase (h)	N/A	N/A	N/A	N/A	N/A	20 ± 0,07	N/A
Sulfite growth rate	N/A	N/A	N/A	N/A	N/A	0,19 ± 0,1	N/A

Sulfite maximum growth	N/A	N/A	N/A	N/A	N/A	0,13 ± 0,01	N/A
OD(600)	141	292	278	135	150	110	77

IsoHop and ethanol alone are not effective preservatives. In some cases, they even enhanced yeast growth. From the preservatives studied in this thesis, benzoate had a more variable effect on yeasts. From the most common contaminants, it only completely inhibited the growth of *W. anomalus.* On average, it halved the activity of the yeasts. Thus, at least for these studied yeasts, benzoate is not a very effective preservative. Sorbate and especially sulfite, were generally the best preservatives. Table 6 below shows for the recommended preservatives for different yeasts for their growth inhibition, based on this experiment. Recommended preservatives have been marked with the symbol +, whereas those that are not recommended have been marked with the symbol -. *S. ludwigii* was the only yeast that was not completely inhibited by any preservative.

Table 6. . Recommended preservatives to use to inhibit yeasts growth, based on this experiment. The symbol + indicates that preservative is recommended, while the symbol - indicates that it is not recommend.

Genus	***Species***	***Code***	***Benzoate***	***Sorbate***	***Sulfite***
Babjeviella	*inositovara*	100-1	+	+	+
Brettanomyces	*anomalus*	C-75001T	-	-	+
	bruxellensis	C-05796	-	-	+
	bruxellensis	WLP650	-	-	+
Candida	*zemplinina*	C-181019	-	+	+
Cyberlindnera	*fabianii*		-	+	+
Hanseniaspora	*uvarum*	FA4	+	+	+
Kazachstania	*servazzi*		-	+	+
Kluyveromyces	*marxianus*	Km2	-	+	+
Lanchea	*fermentati*	C-09854T	-	+	-
	thermotolerans	C-16989	+	+	+
Metschnikowia	*sinensis*	FA7	+	+	+
Mrakia	*gelida*	4GW184	+	+	+
Pichia	*fermentans*		-	+	+

	kluyveri	C-00354	-	-	+
Saccharomyces	*cerevisiae*	WLP380	-	+	+
	cerevisiae var. diastaticus	Belle Saison	-	-	+
	cerevisiae var. diastaticus	C-70060	-	+	+
	pastorianus	W34/70	-	-	+
	paradoxus	C-16968	-	-	+
Saccharomycodes	*ludwigii*		-	-	-
Torulaspora	*delbrueckii*	8bA13	-	+	+
Wickerhamomyces	*anomalus*	C-74021T	+	+	+
Zygosaccharomyces	*rouxii*	C-94197T	-	+	-
Zygotorulaspora	*florentina*	OTA	-	+	-

Combining sorbate and sulfite would provide a secure effect in stopping and inhibiting various yeast contaminations. However, due to sulfite hypersensitivity to asthmatics, the customer base may narrow [30, p. 11]. Also, sulfite amount in this experiment is too high for beer and NABLAB beers [29]. More experiments are needed if sulfite is wanted to use in beer. It must be remembered that preservatives are intended to prolong the shelf life of the product. They do not exclude the importance of ensuring the hygiene of production.

6.4 Temperature

The most important microbial environmental condition is temperature. Temperature plays a major role in the ability of yeasts to grow and maintain their function [26, p.110.] This experiment concerned the yeasts' ability to grow in refrigerator and cold storage temperatures, as well as their ability to grow at 37 °C. Ability to grow at 37 °C determines the risk of yeast infections in humans. Some of the studied yeasts are known to cause yeast infections, such as the top-fermenting yeast *S. cerevisiae* itself. [23, p. 740.] Although yeasts cannot be eliminated under cold conditions, their growth and function usually slow down or stop completely, except psychrophilic yeasts. Temperature is also very important for the shelf life of the product; therefore, it is important to retain the cold chain

of the product. [26, p.110.] Different growth conditions were 37 °C for 3 days, 4 °C for 2 weeks, and 1 °C for 3 weeks (Table 7). There were four different dilutions of the yeasts.

Table 7. All yeasts' growth at 37 °C for 3 days, 4 °C for 2 weeks and 1 °C for 3 weeks. +: positive -: negative w: weak.

Yeast	Growth in 37 °C for 3 days	Growth in 4 °C for 2 weeks	Growth in 1 °C for 3 weeks
B. inositovara	-	+	++
B. anomalus	w	w	-
***B. bruxellensis* (C-05796)**	w	+/w	-
***B. bruxellensis* (WLP650)**	w	-	-
C. zemplinina	-	-	-
C. fabianii	++	-	-
H. uvarum	-	w	-
K. servazzi	-	++	++
K. marxianus	+++	+	w/-
L. fermentati	++	-	-
L. thermotolerans	w	w	w/-
M. sinensis	-	++	+
M. gelida	-	++	+++
P. fermentans	w	++	+
P. kluyveri	+	++	++
S. cerevisiae	+	+/w	w/-
***S. cerevisiae var. diastaticus* (Belle Saison)**	++	w	-
***S. cerevisiae var. diastaticus* (C-70060)**	+	+/w	-
S. paradoxus	w	+/w	-
S. pastorianus	-	+/w	-
S. ludwigii	w	-	-
T. delbrueckii	-	+/w	w/-

W. anomalus	+	+/w	w/-
Z. rouxii	w	-	w/-
Z. florentina	-	+/w	w/-

Most of the yeast did not grow well at 37 °C. 9 yeast of 25, for example, *B. inositovara*, *K. servazzi* and *S. pastorianus,* did not grow at 37 °C (Image 5, Table 7). Weak growth was seen with 8 yeasts, for example, *B. anomalus*, *L. thermotolerans* and *Z. rouxii* (Table Y). Showing moderate growth were *P. kluyveri*, *S. cerevisiae*, *S. cerevisiae var. diastaticus* (C-70060) and *W. anomalus*. Growing well were *C. fabianii*, *L. fermentati* and *S. cerevisiae var. diastaticus* (Belle Saison). (Table 7) *K. marxianus* grew really well at 37 °C and it had clearly more growth than other yeasts, at any temperature (Image 4).

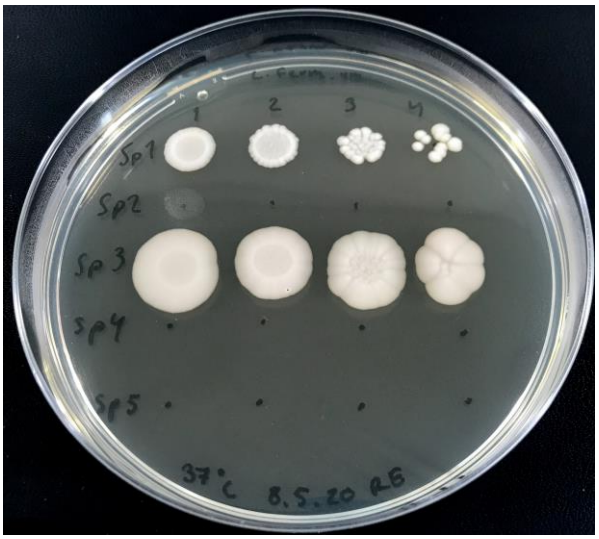


Image 5. Growths at 37°C for 3 days of Sp1: *L. fermentati*, Sp2: *L. thermotolerans*, Sp3: *K. marxianus*, Sp4: *K. servazzi* and Sp5: *S. pastorianus.*

At 4 °C, *B. bruxellensis* (WLP650), *C. zemplinina*, *C. fabianii*, *L. fermentati*, *S. ludwigii* and *Z. rouxii* did not grow. Most yeasts, for example, *H. uvarum*, *L. thermotolerans* and *Z. florentina* (Table 7) grew weakly at this temperature. *K. marxianus* grew moderately at 4 °C. *B. inositovara* grew well. The yeast strains showing strong growth were *K. servazzi*, *M. sinensis*, *M. gelida, P. fermentans* and *P. kluyveri* (Image 6).

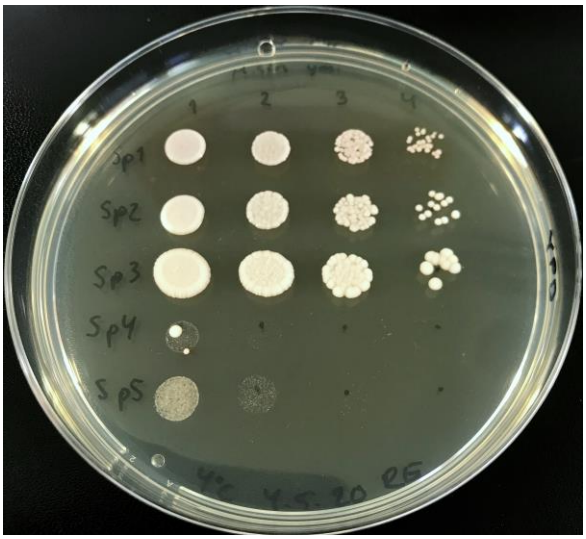


Image 6. Growths at 4°C for 2 weeks of Sp1: *M. sinensis*, Sp2: *P. fermentans*, Sp3: *P. kluyveri*, Sp4: *S. cerevisiae* and Sp5: *S. pastorianus*.

At 1 °C, most of the yeasts did not grow. These included, for example, *C. fabianii*, *K. marxianus*, *S. pastorianus* and *W. anomalus* (Table 7). Very weak growth was seen with *S. cerevisiae* and *Z. florentina*. Moderate growth was seen with *M. sinensis* and *P. fermentans* at 1°C (Image 7). *B. inositovara*, *K. servazzi* and *P. kluyveri*, grew well, while *M. gelida* grew really well at 1 °C (Image 7). *M. gelida* is a known psychrophilic yeast, and its maximum temperature for growth is approximately 17 °C [38, p. 1508].

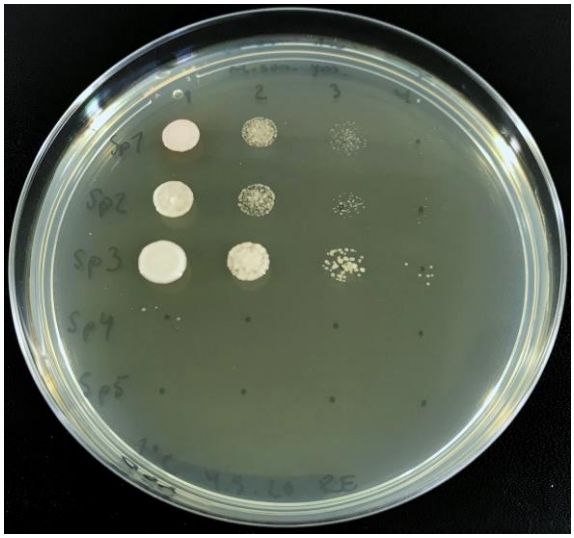


Image 7. Growths at 1°C for 3 weeks of Sp1: *M.sinensis*, Sp2: *P.fermentans*, Sp3: *P.kluyveri*, Sp4: *S.cerevisiae* and Sp5: *S.pastorianus*.

Most of the yeasts did not grow at cold storage temperature, nor at 37 °C. On the other hand, at the refrigerator temperature, most of the yeast thrived. At 4 ° C, only 6 of 25 yeast were unable to grow. Mere refrigerator storage does not prevent contaminant yeasts from spoiling the products, it just happens more slowly. *C. fabianii* did not grow at either cold temperature, suggesting its effectiveness as a contaminant under cold storage conditions is limited. *C. zemplinina* did not grow at any temperature, which is abnormal for this yeast, at least at under cold conditions. Perhaps growth conditions, such as nutrients, were not adequate for *C. zemplinina* in this test. This yeast tolerates even high concentrations of sugar and low temperatures. [37.] On the basis of this experiment, most of the yeasts do not pose a risk to humans. However, some yeast infections can also occur at 35 degrees, such as on skin surfaces; therefore, this experiment does not rule out the chance of the infection [42, p. 538-539]. For a more detailed conclusion, more tests would be required.

7 Conclusion

In this final project in engineering, 25 different yeasts were studied, most of which are non-conventional yeasts as well as known contaminants in the food and beverage industry. The fact that yeast is a contaminant does not exclude its potential for various industrial applications. As long as yeast can be managed and its operating principles understood, it is safe to use. [2.] This study aimed to determine whether these yeasts would be safe to use in brewing and how they could be managed. Some yeast species have already shown their potential in alternative methods for the production of various beers. These included *B. bruxellensis* and *L. thermotolerans* [2]. Traditional brewer's yeasts were here used as a reference to compare the differences between them and non-conventional yeasts. The production of yeast biofilm, disinfectant resistance, tolerance to different preservatives, and the ability to grow at different temperatures were studied. All these properties affect the processing of yeasts in production. The better they produce biofilm, grow at different temperatures and tolerate preservatives as well as disinfectants, the more challenging it is to manage potential contamination by yeasts. [24; 25.]

On the basis of the results, most of the yeasts would be manageable and did not significantly differ from brewer's yeast; thus, they could be safe to use in alternative brewing. Of all the yeasts tested, *K. servazzi* was the most challenging yeast strain to control. It produced a large amount of biofilm, withstood disinfectant exposure, especially in dirty conditions, and grew well at cold storage temperatures. Its use in the production of beer is the riskiest of all these yeast strains. On the basis of the results, it cannot be recommended for production premises without extreme precision and cleanliness. On the positive side, the yeast did not withstand sorbate or sulfate; thus, at least in post-contamination situations where preservatives are used, it would probably not pose any problems. *C. fabianii* produced abundant biofilm and has been found to cause health hazards; therefore, its use in production is risky without further research. The preservatives sulfate and sorbate completely inhibited its growth. On the basis of these experiments alone, its

use cannot be recommended. *B. bruxellensis* (C-05796) can cause production difficulties due to its good biofilm yield and disinfectant tolerance. However, it must be noted, that only one disinfectant was tested in the experiment (P3-oxonia). *B. bruxellensis* (C-05796) also grow moderately at 37, and 4 degrees and only sulfite completely inhibited its growth. *B. bruxellensis* (WLP650) would be experimentally more manageable and a preferred alternative for production. Using *P. kluyveri*, great care should also be taken concerning production hygiene. It produced biofilm well, grew well at different temperatures, and survived sorbate. It could not withstand P3-oxonia; thus, it could be relatively easy to eliminate, but the yeast would require further research on its safety. The experiments with *C. zemplinina* and *M. gelida* should be repeated at more suitable temperatures to obtain more accurate results. Both are cold-tolerant, and the experiments did not allow them to be set at suitable temperatures in biofilm production or preservative tests. However, *C. zemplinina* grew in the preservative test. *C. zemplinina* also did not grow in the temperature test, which is distinct at cold temperatures. Especially since the temperature test was performed at the same time as the preservative test. Thus, the possibility that the yeast may have lost its function in the inoculation dish cannot be excluded, which may have been part of the reason for the lack of growth in disinfectant and biofilm experiments.

New experiments should be done especially for those yeasts that produced large amount of biofilm. The experiments could at least test their resistance to detergents in biofilm form. Yeasts that grew at 37 degrees and/or have a background of adverse health effects should also be evaluated for their clinical importance, for example, by antibiotic testing.

For new yeasts to be used safely in production, their use and development should be monitored, and adequate hygiene ensured. When working with microbes, it should be remembered that while the surface looks clean, it may not be microbiologically clean. Without proper knowledge of hygiene principles, contamination management is not possible. [4, p. 366.]

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Appendix 1. Growths with preservatives

Table 1.1 Rest of the yeasts growths with different conditions (control, IsoHop, benzoate, ethanol, sulfite and sorbate).

Yeast	*B. inositovara*	*B. anomalus*	*B. bruxellensis* (C-05796)	*B. bruxellensis* (WLP650)	*C. zemplinina*	*C. fabianii*
Control lag phase (h)	23 ± 0,04	7,5 ± 0,07	5 ± 0,05	16 ± 0,1	6 ± 0,03	8 ± 0,03
Control growth rate	0,05 ± 0,07	0,18 ± 0,07	0,22 ± 0,04	0,08 ± 0,1	0,2 ± 0,05	0,2 ± 0,04
Control maximum growth	0,05 ± 0,003	0,16 ± 0,008	0,16 ± 0,007	0,08 ± 0,007	0,2 ± 0,009	0,15 ± 0,01
IsoHop lag phase (h)	11,5 ± 0,09	8 ± 0,11	5 ± 0,02	18 ± 0,03	9 ± 0,03	5,5 ± 0,02
IsoHop growth rate	0,04 ± 0,2	0,21 ± 0,1	0,20 ± 0,02	0,09 ± 0,05	0,2 ± 0,05	0,18 ± 0,03
IsoHop maximum growth	0,06 ± 0,005	0,15 ± 0,007	0,15 ± 0,007	0,08 ± 0,004	0,16 ± 0,006	0,14 ± 0,005
Ethanol lag phase (h)	N/A	10 ± 0,08	8 ± 0,02	18 ± 0,02	13,5 ± 0,03	8,5 ± 0,02
Ethanol growth rate	N/A	0,09 ± 0,1	0,17 ± 0,03	0,08 ± 0,06	0,15 ± 0,05	0,17 ± 0,02
Ethanol maximum growth	N/A	0,07 ± 0,009	0,13 ± 0,006	0,06 ± 0,005	0,14 ± 0,007	0,13 ± 0,005
Benzoate lag phase (h)	N/A	16,5 ± 0,15	29,5 ± 0,05	35 ± 0,002	14 ± 0,08	40,5 ± 0,001
Benzoate growth rate	N/A	0,14 ± 0,2	0,03 ± 0,2	0,006 ± 0,02	0,1 ± 0,2	0,02 ± 0,01
Benzoate maximum growth	N/A	0,1 ± 0,01	0,017 ± 0,003	0,002 ± 0,0008	0,13 ± 0,01	0,004 ± 0,001

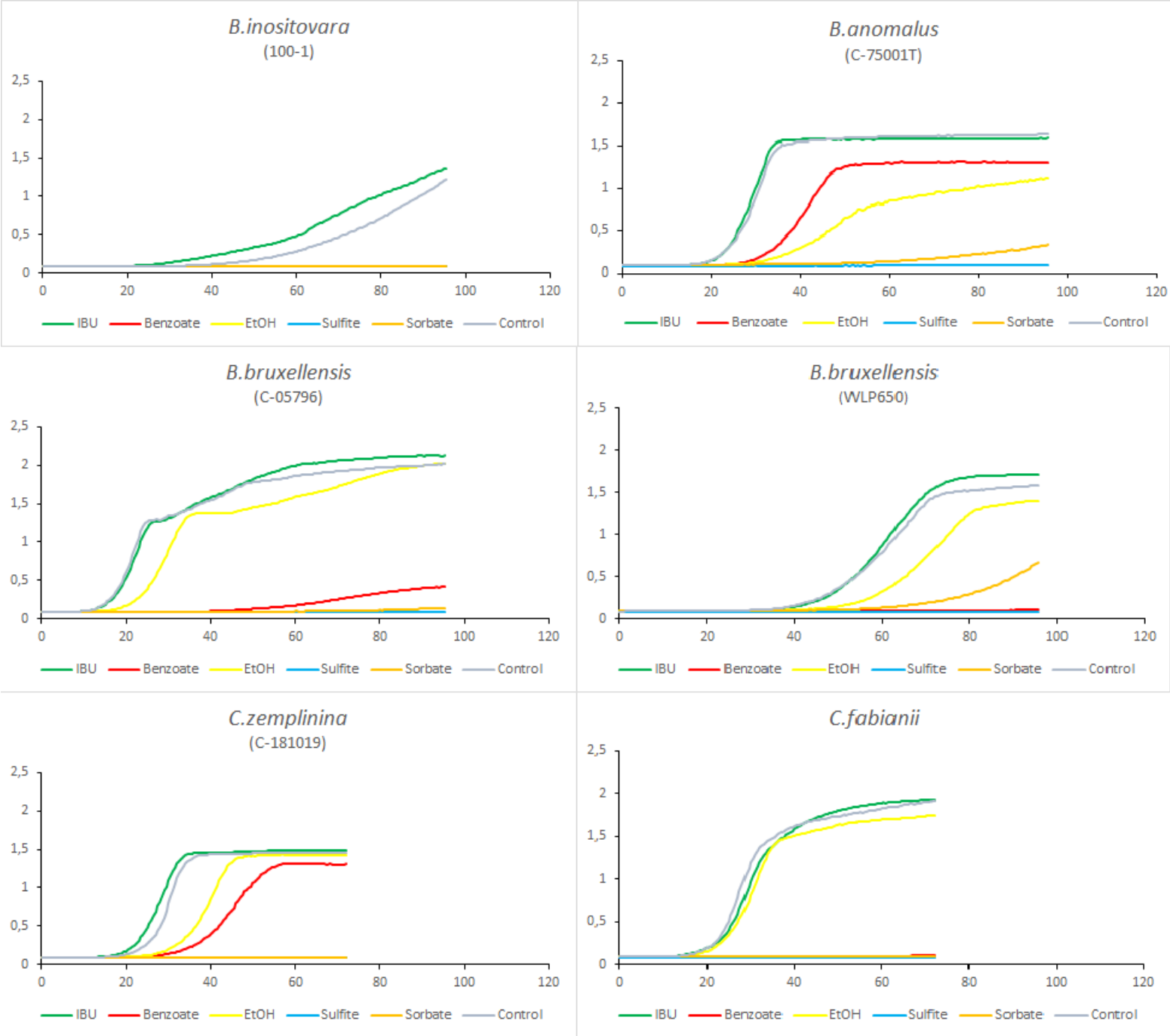
Sorbate lag phase (h)	N/A	23 ± 0,01	59 ± 0,004	19 ± 0,007	N/A	N/A
Sorbate growth rate	N/A	0,021 ± 0,07	0,017 ± 0,04	0,05 ± 0,04	N/A	N/A
Sorbate maximum growth	N/A	0,03 ± 0,004	0,007 ± 0,002	0,04 ± 0,003	N/A	N/A
Sulfite lag phase (h)	N/A	N/A	N/A	N/A	N/A	N/A
Sulfite growth rate	N/A	N/A	N/A	N/A	N/A	N/A
Sulfite maximum growth	N/A	N/A	N/A	N/A	N/A	N/A
OD(600)	73,2	127	111	119	162	115
Yeast	***H. uvarum***	***L. fermentati***	***L. thermotolerans***	***M. sinensis***	***M. gelida***	***P. fermentans***
Control lag phase (h)	4,5 ± 0,07	3 ± 0,07	3,5 ± 0,03	6 ± 0,04	18 ± 0,002	5,5 ± 0,01
Control growth rate	0,30 ± 0,1	0,26 ± 0,05	0,22 ± 0,03	0,15 ± 0,04	0,01 ± 0,01	0,24 ± 0,02
Control maximum growth	0,19 ± 0,02	0,19 ± 0,006	0,19 ± 0,003	0,17 ± 0,005	0,02 ± 0,001	0,16 ± 0,003
IsoHop lag phase (h)	4,5 ± 0,02	5 ± ,0,05	4 ± 0,04	5 ± 0,04	N/A	6 ± 0,03
IsoHop growth rate	0,33 ± 0,03	0,26 ± 0,04	0,25 ± 0,03	0,17 ± 0,04	N/A	0,30 ± 0,02
IsoHop maximum growth	0,22 ± 0,009	0,2 ± 0,005	0,19 ± 0,003	0,16 ± 0,007	N/A	0,17 ± 0,005
Ethanol lag phase (h)	8 ± 0,06	7 ± 0,05	7 ± 0,02	13 ± 0,06	N/A	6 ± 0,02
Ethanol growth rate	0,22 ± 0,08	0,23 ± 0,05	0,22 ± 0,02	0,11 ± 0,07	N/A	0,21 ± 0,02
Ethanol maximum growth	0,15 ± 0,009	0,18 ± 0,005	0,18 ± 0,003	0,04 ± 0,007	N/A	0,17 ± 0,007

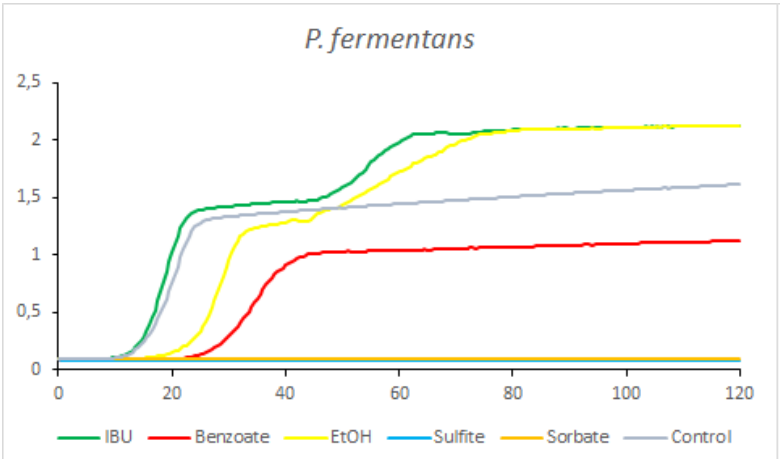
Benzoate lag phase (h)	N/A	13 ± 0,03	N/A	N/A	N/A	11,5 ± 0,05
Benzoate growth rate	N/A	0,14 ± 0,3	N/A	N/A	N/A	0,16 ± 0,09
Benzoate maximum growth	N/A	0,12 ± 0,006	N/A	N/A	N/A	0,08 ± 0,009
Sorbate lag phase (h)	N/A	N/A	N/A	N/A	N/A	N/A
Sorbate growth rate	N/A	N/A	N/A	N/A	N/A	N/A
Sorbate maximum growth	N/A	N/A	N/A	N/A	N/A	N/A
Sulfite lag phase (h)	N/A	34,5 ± 0,16	N/A	N/A	N/A	N/A
Sulfite growth rate	N/A	0,17 ± 0,2	N/A	N/A	N/A	N/A
Sulfite maximum growth	N/A	0,13 ± 0,02	N/A	N/A	N/A	N/A
OD(600)	140	177	269	120	157	69
Yeast	***S. cerevisiae var.diastaticus* (Belle Saison)**	***S. cerevisiae var.diastaticus* (C-70060)**	***S. paradoxus***	***T. delbrueckii***	***Z. rouxii***	***Z. florentina***
Control lag phase (h)	3,5 ± 0,05	4 ± 0,1	4 ± 0,008	5,5 ± 0,02	7 ± 0,07	5 ± 0,02
Control growth rate	0,25 ± 0,06	0,24 ± 0,1	0,28 ± 0,01	0,23 ± 0,02	0,11 ± 0,1	0,19 ± 0,03
Control maximum growth	0,26 ± 0,01	0,25 ± 0,03	0,19 ± 0,006	0,16 ± 0,006	0,1 ± 0,006	0,17 ± 0,005
IsoHop lag phase (h)	5 ± 0,08	4 ± 0,06	4,5 ± 0,02	4 ± 0,03	7 ± 0,05	5,5 ± 0,05
IsoHop growth rate	0,27 ± 0,07	0,31 ± 0,06	0,30 ± 0,02	0,25 ± 0,02	0,10 ± 0,09	0,20 ± 0,04

IsoHop maximum growth	0,2 ± 0,008	0,26 ± 0,008	0,17 ± 0,003	0,15 ± 0,003	0,07 ± 0,005	0,17 ± 0,004
Ethanol lag phase (h)	7,5 ± 0,008	8 ± 0,05	7 ± 0,06	12 ± 0,17	21 ± 0,07	9,5 ± 0,07
Ethanol growth rate	0,28 ± 0,01	0,33 ± 0,07	0,30 ± 0,06	0,12 ± 0,2	0,13 ± 0,1	0,07 ± 0,08
Ethanol maximum growth	0,18 ± 0,003	0,22 ± 0,007	0,18 ± 0,007	0,1 ± 0,01	0,09 ± 0,006	0,07 ± 0,08
Benzoate lag phase (h)	13,5 ± 0,16	26 ± 0,1	16,5 ± 0,13	19,5 ± 0,16	20 ± 0,04	45 ± 0,1
Benzoate growth rate	0,19 ± 0,07	0,16 ± 0,2	0,13 ± 0,2	0,09 ± 0,3	0,04 ± 0,1	0,08 ± 0,2
Benzoate maximum growth	0,11 ± 0,004	0,12 ± 0,02	0,1 ± 0,02	0,08 ± 0,1	0,04 ± 0,005	0,09 ± 0,009
Sorbate lag phase (h)	17 ± 0,009	N/A	25 ± 0,04	N/A	N/A	N/A
Sorbate growth rate	0,02 ± 0,07	N/A	0,04 ± 0,2	N/A	N/A	N/A
Sorbate maximum growth	0,005 ± 0,002	N/A	0,04 ± 0,008	N/A	N/A	N/A
Sulfite lag phase (h)	N/A	N/A	N/A	N/A	26 ± 0,04	46 ± 0,07
Sulfite growth rate	N/A	N/A	N/A	N/A	0,11 ± 0,06	0,17 ± 0,1
Sulfite maximum growth	N/A	N/A	N/A	N/A	0,10 ± 0,006	0,14 ± 0,01
OD(600)	111	141	128	253	179	262

Appendix 2. Growth curves with preservatives

Figure 2.1 Rest of the growth curves with IsoHop (IBU), benzoate, ethanol, sulfite, sorbate and control. The x-axis indicates time, and the y-axis shows the yeast growth.

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Benzoate
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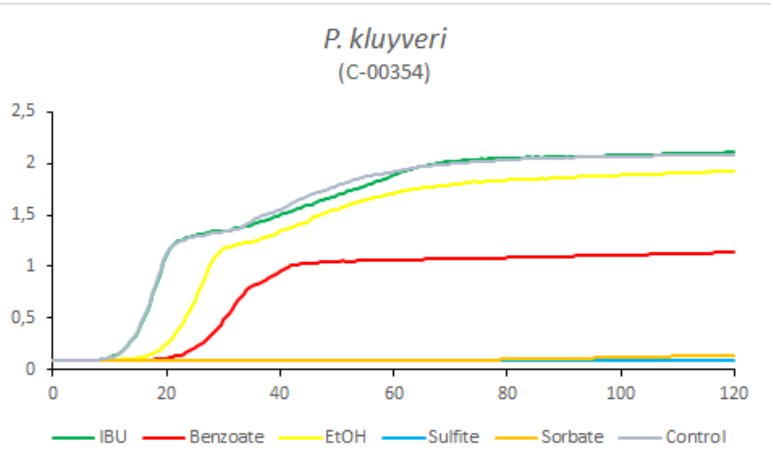
Sorbate
Control


P. kluyveri
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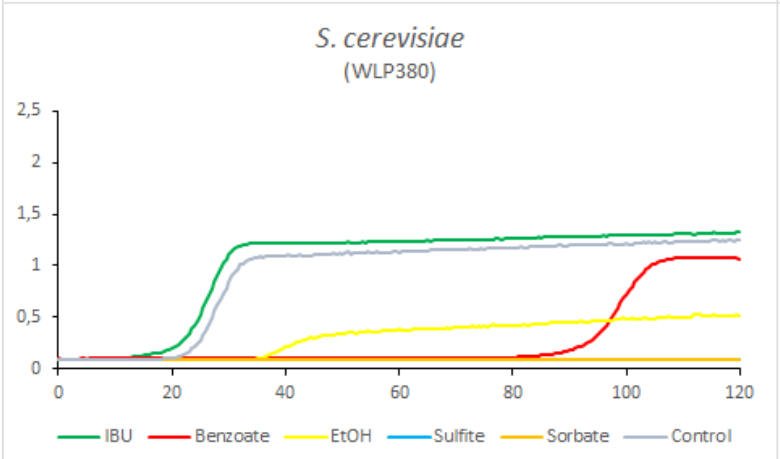
S. cerevisiae
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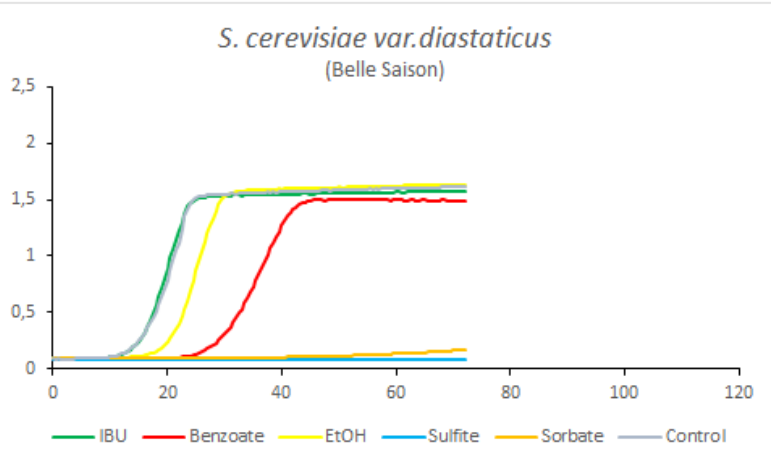
S. cerevisiae var.diastaticus
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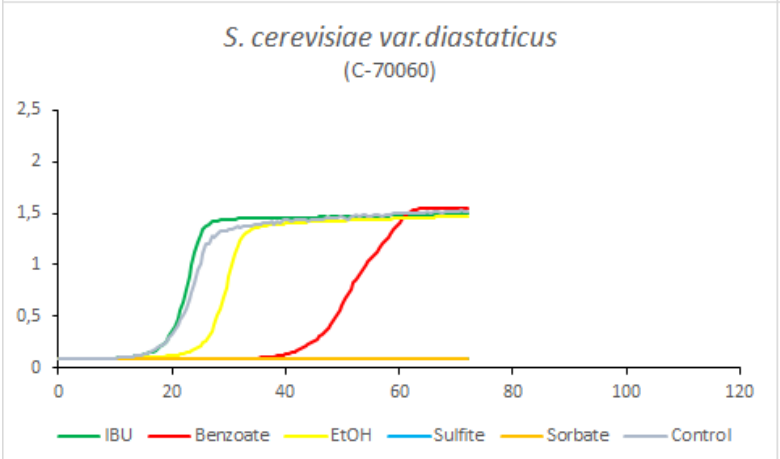
S. cerevisiae var.diastaticus
(C-70060)
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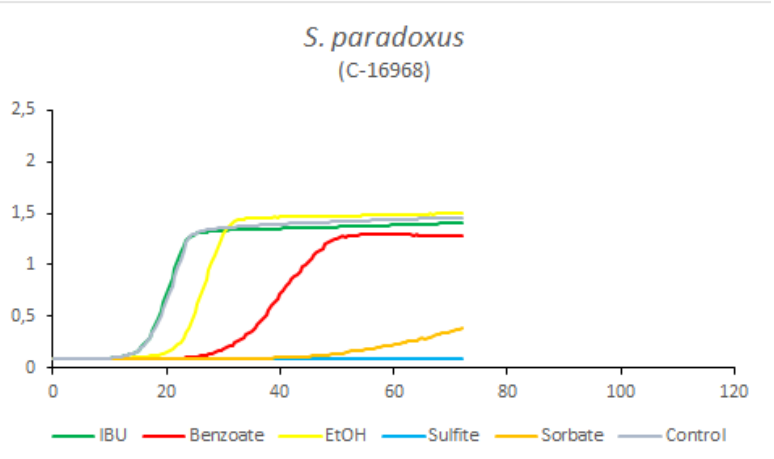
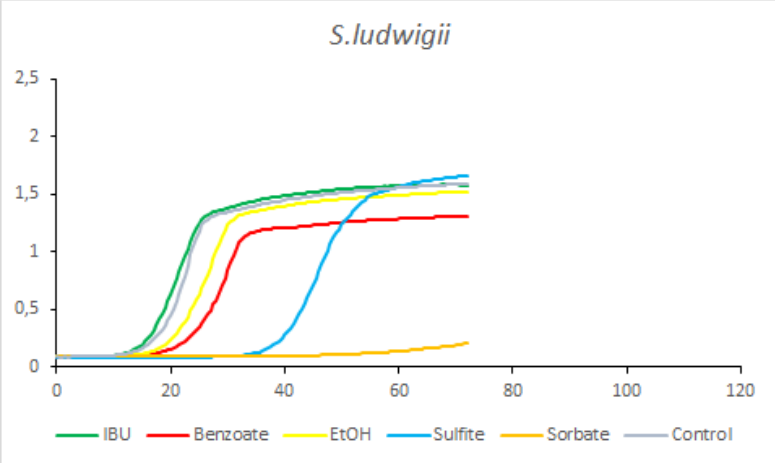
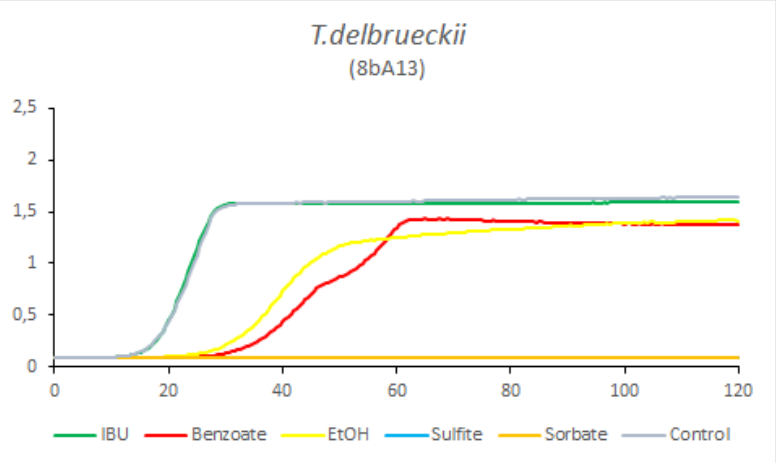
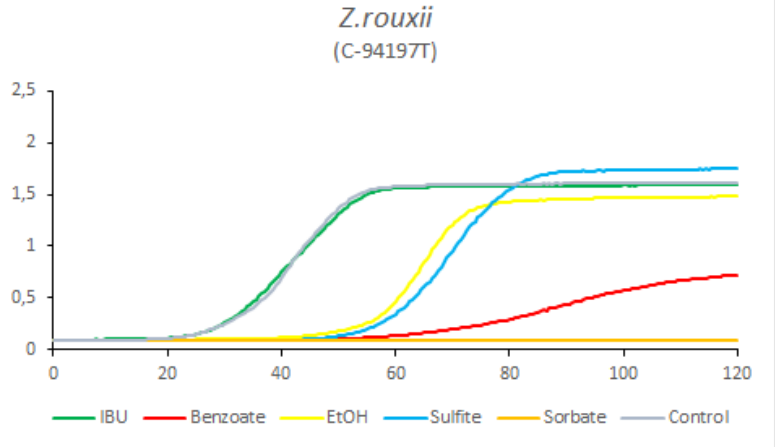
120
IBU
Benzoate
EtOH
Sulfite
Sorbate
Control


S. paradoxus
(C-16968)
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
IBU
Benzoate
EtOH
Sulfite
Sorbate
Control


S.ludwigii
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
IBU
Benzoate
EtOH
Sulfite
Sorbate
Control


T.delbrueckii
(8bA13)
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
IBU
Benzoate
EtOH
Sulfite
Sorbate
Control


Z.rouxii
(C-94197T)
2,5
2
1,5
1
0,5
0
0
20
40
60
80
100
120
IBU
Benzoate
EtOH
Sulfite
Sorbate
Control