THE INFLUENCE OF POTASSIUM SORBATE ON THE GROWTH OF *CANDIDA GUILLIERMONDII* IN OIL-WATER SYSTEMS AT VARYING pH VALUES AT 7°C AND 22°C.

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Preface

This thesis focused on modelling the growth of *Candida guilliermondii* in different matrices with and without potassium sorbate at 22°C and 7°C. It was a privilege to be part of this study that will generate a lot of knowledge especially for the yellow fat spread industry. Firstly I would like to thank my Heavenly Father who gave me strength everyday to work hard and accomplish the work in this book. Secondly, I am sincerely thankful to Prof. Frank Devlieghere, Dr. An Vermeulen, Miss Šoljić Irena, Ann Dirckx and the staff at the Laboratory of Food Microbiology and Food preservation who all tirelessly provided guidance and help whenever I needed it. Last but not least, I cannot forget to thank my family (Mutumba/Kangave) back home in Uganda and the Decorte / Van Maele family here who were all a source of encouragement.

Catherine Nambooze

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5.1 CONCLUSION

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ANNEX 1
List of abbreviations

CFU - Colony forming units

ComBase - Combined Database

EC - European Commission

EU - European Union

FAO – Food and Agricultural Organization

FDA-Food and Drug Authority

GRAS - Generally Regarded As Safe

GT - Generation time

HCl - Hydrochloric acid

ICMSF- International Commission on Microbiological Specifications for Foods

KS- Potassium sorbate

mL- milliliters

NaOH – Sodium hydroxide

PPS – Peptone physiological salt

Rpm – Revolutions per minute

YNB – Yeast Nitrogen Base (without amino acids)
Abstract

The food industry is now facing enormous challenges in controlling microbial growth as consumer market trends reflect a growing demand for less heavily processed foods. With minimum processing comes an increase in diversity of spoilage species and consequently spoilage susceptibility. The yeast *Candida guilliermondii* has been isolated in butter, buttermilk, olives, olive oil, yoghurt, beer, fish, to mention but a few. This study aimed at characterizing the effect of pH, potassium sorbate, addition of sunflower oil/INES 48 hardstock representing solid fat and temperature on the growth of *Candida guilliermondii*. To this effect, the yeast was grown in Yeast Nitrogen Base broth (YNB) at varying pH levels (3.5, 4.5, 5.5, and 6.5) with and without addition of potassium sorbate (100 ppm) at 7°C and 22°C. In addition to this, oil-water systems with liquid oil and solid fat were also studied at the above conditions. pH had a significant effect (p < 0.05) on the growth rate of *Candida guilliermondii* with the lowest growth rate achieved at pH 3.5 at both 7°C and 22°C. With addition of liquid oil, there was no significant difference (p >0.05) observed between the generation times for all pH levels at these temperatures. Slightly higher growth rates were observed with sunflower oil addition than in the matrix without oil, especially at the low pH levels. With addition of solid fat, similar generation times were observed among the pH levels. *Candida guilliermondii* exhibited the highest growth rates in the matrix with solid fat when compared to the other matrices with oil or with only broth at 7°C and 22°C. In the experiments with sorbic acid in YNB broth, sorbic acid presented significant effects (p <0.05) on the growth of *Candida guilliermondii* especially at the low pH levels 3.5 and 4.5 where total inhibition was observed. In the oil matrix, complete inhibition was not observed at 22°C but significant differences (p <0.05) were observed between pH 3.5 and the rest of the pH levels. Generation times in the solid fat matrix in the presence of sorbic acid were shorter at all pH levels than those observed in the liquid oil matrix. At 7°C however, there was complete inhibition at pH 4.5 for the solid fat matrix. Results from this study are expected to contribute to the limited work available on how spoilage yeasts behave in oil-water systems.
1 INTRODUCTION
1.1 BACKGROUND

Present day consumer market trends reflect a rapidly growing demand for convenient, high quality foods that are less heavily processed and have a competitive price. This consumer pressure is passed along the supply chain to the food industry which is now facing enormous challenges on controlling microorganisms. This is because with minimum processing comes an increase in diversity of spoilage species and consequently spoilage susceptibility (Membré et al., 2016). The cost of microbiological safety issues is of economic importance to the food industry and ranges from having a high level of rework/ product disposal, product recalls, negative product image and sometimes sickness or death if pathogens are involved. The industry can therefore compromise with some quality aspects such as colour but never with food safety. This makes food safety the first priority of food production and preservation.

Aside from food safety, the heart of food science and technology is food processing and preservation to produce value added products and increase storage life. Food by its very nature is expected to be nutritious and is therefore a rich habitat for microorganisms like bacteria, yeasts and moulds. Generally speaking however, it appears that fungal metabolism is best suited to substrates high in carbohydrates, while bacteria are more likely to spoil proteinaceous foods (Pitt et al., 1997). The growth of yeasts and moulds is generally slower than that of bacteria but the wide variety of ecological niches they can exploit; their ability to utilize a variety of substrates and tolerance of more extreme conditions than bacteria makes them formidable spoilage agents (Huis in’t Veld, 1996). Examples of food spoilage yeasts belong to the Saccharomyces species, Candida species, Debaryomyces species, Rhodotorula species, Pichia species and many others. Foods that are commonly affected by yeast include fruit juices, sugar and flavor syrups, confectionery products, vegetable salads with acid dressings, fermented dairy products to mention but a few. Yeasts are known to cause swelling/ explosion of containers due to the production of excess gas. Aside from that, they also cause cloudiness or development of particulates in beverages and sometimes off-flavours. Romano et al., 1999 reported that off flavours developed due to high levels of acetoin and acetaldehyde by Saccharomycodes ludwiggii in fruit juice. In some cases, addition of preservatives against yeasts may enhance the production of off flavours. A study on high sugar foods by Casas et al., 1999 showed that degradation of sorbic acid to 1,3-
pentadiene by *Zygosaccharomyces rouxii* lead to petroleum-like off odor. Other indications of spoilage include surface discoloration, slimy surface, free fatty acid rancidity etc.

The genus *Candida* encompasses about 200 species of yeasts (Segal *et al.*, 1994) and makes up one quarter of all known yeasts (Blackburn, 2006). The yeast *Candida guilliermondii* has been isolated in dairy products, fish, soft drinks, butter, and in the environment. It has both positive and negative implications in the food industry that will be discussed in a latter chapter. However recent studies show that this yeast is a subject of emerging interest in public health as an opportunistic pathogen (Satyanarayana *et al.*, 2005). This raises concern about its presence not only as a potential pathogen but also as food spoilage yeast and puts a focus on ways to control its growth.

In the modern and highly automated food processing industry, the ability to detect the onset or source of microbial spoilage is particularly advantageous. At present most food microbiology laboratories identify spoilage yeasts using phenotype based methods or traditional colony count methods which can be laborious and time consuming. These downsides have driven a number of attempts to develop rapid detection and enumeration methods. Some of the technologies include immunological techniques, molecular methods and automation of the traditional methods such as the use of the spiral plating technique. However detection alone is not enough, the food industry is constantly challenged with ways to eliminate or control these microorganisms.

The preservation and processing of food is not as simple and it is critical to understand the effect of each preservation method on the food and the microorganism. The most common physical preservation treatment is the use of temperature which is one of the factors that are varied in this study. Low temperature treatments include refrigeration and freezing which in contrast to heat do not destroy enzymes and microorganisms to any significant extent but merely retard their activity (Berk, 2013). Such retardation remains in place only as long as the food is not exposed to temperature abuse.

Another fundamental technique in food preservation is the use of chemical preservatives. Preservatives for use in foods must be safe for human consumption, have a wide range of anti-microbial activity, and should not affect the sensory qualities of the product. Under this
constraint, food technologists are limited to the use of weak acid preservatives like benzoic acid, sorbic acid, acetic acid etc. These usually work best at low pH levels. Sorbic acid in particular is effective in preventing mold and yeast growth (Sperber, 2009) but since it has low water solubility, it is usually applied in the form of its salt derivatives. Common foods where it is used include cheeses, bakery products, fruit juices, beverages, salad dressings etc.

It is worth noting that many people are more careful and skeptical about additives added to food. This is due to the negative image that some additives have or individual sensitivities that lead to allergic reactions. Food industries are compelled to reduce quantities or totally remove additives which has its disadvantages. Arroyo et al., 2005 reported that the use of suboptimal concentrations of preservatives such as organic acids may stimulate the growth of spoilage fungi including *Hyphopichia burtonii*, *Candida guilliermondii* and *Aspergillus flavus*. It has also become evident that microorganisms are continuously adapting to survive in the presence of previously effective control methods (Rico et al., 2008).

A number of new preservation techniques have thus been developed to satisfy current demands for economic preservation. The most common method involves harmonizing of food preservation techniques through hurdle technology. Hurdle technology achieves synergies through the combined manipulation of physico-chemical conditions (temperature, water activity, redox potential, acidity and preservatives), which while they may not prevent the growth of pathogens or spoilage microorganisms on their own, they will do so collectively (Ohlsson et al., 2000).

Other possible new food preservation techniques include the use of ultra high pressure, high intensity light, pulsed electric fields, antimicrobial essential oils, and irradiation. However any new technique is likely to select for extremophilic yeast species which are able to survive the treatment (Querol et al., 2006). Ultraviolet light is known to damage the yeast DNA bringing about lethal mutation. Using full-spectrum light with intensities of 0.1-0.4 J/cm² for only 2-4 flashes reduces yeast cell counts by five to six log cycles.
1.2 PROBLEM STATEMENT

Food preservation has become an increasingly important component of the food industry. With the ever changing consumer preferences, food manufacturers have been able to devise ways to reduce or eliminate spoilage and pathogenic microorganisms like bacteria, yeast, mould etc in order to provide safe and convenient food products. Aside from safety, they also face large economical losses as microbial spoilage accounts for up to 40% of food losses of all food grown for human consumption worldwide (Hazan et al., 2004). Unlike bacteria, yeasts prevail even in very harsh conditions and so are more difficult to control. The yeast *Candida guilliermondii* has been isolated in a number of foods, human infections and the environment. It is well known to exhibit decreased susceptibility to antifungal agents, making it an emerging pathogen and a serious hazard for food processors (Pfaller et al., 2006). Foods where it has been found include olives, olive oil, fish, beer, milk, butter, and buttermilk. It is known to cause undesirable flavours, and texture changes in food, and invasive candidiasis in humans. Many studies on this yeast have been conducted in the clinical microbiology sector but not so much has been done in the food sector.

The food sector has concentrated a lot on yeasts like *Saccharomyces cerevisiae, Zygo. rouxii, Rhodotorula spp* among others but there is very limited work on *Candida guilliermondii*. In addition to this, little information is currently available on combining sub-inhibitory factors to preserve food against the proliferation of this yeast. Such studies are very time consuming and expensive so predictive modeling has been used to test the consequences of a number of factors changing at the same time. Combinations of pH, preservatives and temperature are common factors used in the hurdle approach to preservation in the food industry. Frequently used preservatives include sorbates, benzoates, sulphites, nitrates and parabens. In these experiments the effect of sorbic acid as a preservative is studied. It is important to note that the inhibitory influence of sorbic acid is greatest at or below its pKa value. The pKa is a measure of the strength of an acid in solution. Acids are required in their undissociated form to penetrate the membrane of microorganisms and cause cell death. A study by Rehm et al., 1963 indicated that the dissociated form of weak acids may also show antimicrobial effect, making their action less pH dependant.
The antimicrobial action of sorbic acid increases as the pH value decreases below its pKa value, 4.75. However due to solubility challenges with sorbic acid, its salts like potassium sorbate, sodium sorbate or calcium sorbate are used instead. Of these, potassium sorbate is the primary form used in foods (Hui, 2006). Sodium sorbate leads to slight pseudo-allergic reactions (Food-Info, 2014) and has been reported by Mamur et al., 2012 to have mutagenic and genotoxic effects and may thus cause cancer. Aside from this, Luck (1980) reported that sodium sorbate is only stable for a few weeks and is highly sensitive to oxidation. Calcium sorbate on the other hand is a valuable form of sorbic acid in oil water systems because it is insoluble in fat and is therefore readily available in the aqueous phase to inhibit microbes. However its use is limited because it has a water solubility of 1.2% and may impart an off taste to the food (Davidson et al., 2005). Antifungal agents like diacetyl, phenolic antioxidants, and carbon dioxide have also been used in the industry but have down sides. For example diacetyl imparts a sharp specific odor to the food, carbon dioxide inhibits mold growth but will not prevent growth of yeasts, while some phenolic antioxidants have been shown to stimulate yeast growth at low levels. This study therefore aims at characterizing the growth of *Candida guilliermondii* using a combination of pH, preservative (potassium sorbate) and temperature in systems that mimic table spreads.

1.3 OBJECTIVES OF THE STUDY

The main objectives of this study include:

1) To study the influence of varying pH (3.5, 4.5, 5.5, 6.5) and temperature (7°C and 22°C), representative of pH’s of water-in-oil emulsions and their keeping temperatures, on the growth of *C. guilliermondii*.

2) To study the effect of potassium sorbate, a common preservative in water-in-oil emulsions, at the different pH levels and temperature on the growth parameters of *C. guilliermondii*

3) To investigate the influence of partitioning of sorbic acid at varying pH and temperature in an immiscible broth/sunflower oil matrix on the growth parameters of *C. guilliermondii*

4) To investigate the influence of partitioning of sorbic acid at varying pH and temperature in an immiscible broth/sunflower oil + inES 48 hardstock matrix on the growth parameters of *C. guilliermondii*
2 LITERATURE REVIEW
2. 1 THE YEAST Candida Guilliermondii

2.1.1 Characteristics of Candida guilliermondii

Candida guilliermondii is the designated species name for asporogenous strains of Pichia guilliermondii. Pichia guilliermondii represents a collection of sporogenous strains which formerly were classified as the asporogenous species Candida guilliermondii. This means that each strain of Candida guilliermondii which is able to hybridize with any strain of Pichia guilliermondii must be transferred and considered as Pichia guilliermondii (Satyanarayana et al., 2009).

Candida guilliermondii is an aerobic yeast and thus cannot grow under strict anaerobic conditions. Its optimal growth temperature is 30°C but it can grow at temperatures as low as 7°C provided other environmental parameters like pH are suitable. The yeast also utilizes hydrocarbons as sole carbon and energy source. According to research at the University of Adelaide, the microscopic morphology shows spherical to sub-spherical budding cells of 2.0-4.0 x 3.0-6.5 μm in size (“Mycology online - Candida guilliermondii”, 2016). Appearance on solid agar differs among different agar types for example pale pink-purplish colonies are observed on CHROMagar Candida (Pinoni et al., 2007) while on Sabourand’s dextrose agar colonies are cream coloured, smooth and flat. Shavlovsky et al. (1978) analyzed 147 strains of P. guilliermondii and 146 of these strains were able to excrete a yellow pigment. This is due to the ability of this species to overproduce riboflavin during growth in iron deficient liquid media.

2.1.2 Significance of Candida guilliermondii

Due to its ability to utilize certain carbohydrates, Candida guilliermondii is used as a food additive and is safely used for fermentation in foods like pickles, rinds and fruits. In addition to this, the enzyme system of the viable organism and its concomitant metabolites are actively used in the production of citric acid which is a widely used preservative (Burdock, 1996). Candida guilliermondii is also a xylitol-producing yeast and is known to yield above 0.7g xylitol/g xylose. Xylitol is of great economic interest due to its anti-carcinogenicity and sweetening properties which have been exploited in the production of food stuffs, odontological products and pharmaceuticals (Lima et al., 2003). Candida guilliermondii has
been shown to be active against the main post harvest pathogen *Penicillium digitatum* that causes green rot in citrus fruits. It is therefore applied as a coating on citrus and grape fruits, which offers a great biological control alternative to the usual chemical treatments (McGuire, 1994). Besides the food industry, *Candida guilliermondii* under suitable growth conditions is induced to produce extracellular lipases. This is a cost and time effective way of producing such enzymes which are in turn used in biodiesel production (Oliveira *et al.*, 2014).

However, negative implications of the yeast have also been encountered. Spoilage of fermented milk products like yoghurt and quark has been observed through blowing of the packages and off flavours (fruity, bitterness). Since *Candida guilliermondii* ferments glucose, sucrose, galactose and not lactose (Oguntoyinbo, 2008; “Mycology online-*Candida guilliermondii*”, 2016), spoilage by it occurs after lactose has been hydrolyzed by lactic acid bacteria to produce galactose (Boekhout *et al.*, 2003). Gas swelling caused by this yeast is also predominantly observed in yoghurt types where other sugars like fruit sugars have been added (Wrent *et al.*, 2015). In soft and fresh cheeses, gas production leading to textural changes and flavor defects has been associated with presence of *Candida guilliermondii*. Aside from dairy products, it is responsible for visible/surface spoilage in bread causing white or pink patches. This visible yeast growth is associated with products of high water activity and short shelf life (Legan *et al.*, 1991). *Candida guilliermondii* has also been implicated in spoilage of high sugar foods such as soft drinks as a result of contamination from the general factory environment particularly in areas where sugary products are spilled (Querol *et al.*, 2006).

It is important to note that the strains used in food production should be non pathogenic and non toxicogenic. This is because some strains cause a variety of deep seated infections (fungemia) in cancer patients (leukemia, lymphoma or multiple myeloma), surgical patients and less frequently in IV drug users (Reiss *et al.*, 2012; Jung *et al.*, 2015). It has also been isolated from blood, skin and soft tissue infections. Pfaller *et al.* (2006) reported that this yeast exhibits decreased susceptibility to anti-fungal agents especially fluconazole making it even more difficult to handle. Another case of pathogenicity was observed in animals particularly the Arab mare, where *Candida guilliermondii* caused an abortion leading to economic losses for an equine industry (Stefanetti *et al.*, 2014).
2.2 THE TECHNOLOGY OF TABLE SPREADS

2.2.1 Classification of table spreads

Butter, margarine and spreads are visco-elastic solids that exist in the form of water in oil emulsions i.e. water is the discontinuous phase in the form of droplets dispersed in the continuous phase of oil (Brown, 2008). The most important parameters used to evaluate these products include appearance, texture, spreadability, and chemical or microbial stability. These products may be categorized according to their fat content (%) as shown in Table 2.1.

### Table 2.1. Categories of table spreads

<table>
<thead>
<tr>
<th>Description</th>
<th>Fat content %</th>
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<tr>
<td>Butter/Margarine/Blend</td>
<td>80%-90%</td>
</tr>
<tr>
<td>Three-quarter fat butter/margarine/blend</td>
<td>60%-62%</td>
</tr>
<tr>
<td>Half-fat butter/margarine/blend</td>
<td>39%-41%</td>
</tr>
<tr>
<td>Dairy spread/Fat spread/ Blended spread (X%)</td>
<td>&lt;39%</td>
</tr>
<tr>
<td></td>
<td>41% &lt; X &lt; 60%</td>
</tr>
<tr>
<td></td>
<td>62% &lt; X &lt; 80%</td>
</tr>
</tbody>
</table>

**Source:** EU Council Regulation 1234/2007

The consumer trend towards reduced or low fat spreads is due to people’s increasing awareness and cautiousness towards health and the dangers of high fat intake. These spreads may be based on vegetable oils i.e. (soybean or sunflower oil), a blend of vegetable oil and butterfat or butterfat alone. Depending on the nature of the product, the recipe typically consists of a fat blend, stabilizers, flavours, colours, water, salt, preservatives and an emulsifier. The emulsifier should be able to prevent phase separation and to bind large amounts of water in order to obtain a narrow water droplet distribution, hence a microbiologically stable product (Sinha *et al*., 2009). If water is present in channels or coarse droplets, it is important to be critical of the aqueous phase composition, especially pH and soluble components like sucrose, salt and preservatives. In general pH of yellow fat products ranges from as low as 3.5 to 6.5 (Gunstone *et al*., 1997; van Zijl *et al*., 2000). However the pH of low fat dairy spreads should not be less than 4.5 as this leads to precipitation of
caseinate which lowers its emulsifying properties and affects the physical structure and keeping quality of the spread.

Low fat/reduced fat spreads are produced by shearing at a high speed to achieve water droplet size reduction and simultaneously cooling to entrap the dispersed droplets in a continuous phase of fat (Fox, 1995). The fat phase is composed of different crystal polymorphs $\alpha$, $\beta$, and $\beta^1$ with $\beta$ being the most thermodynamically stable (Akoh, 2006). The kind of crystals present is closely related to the desirable properties of the spreads. Sinha et al., 2009 reported that fats containing at least 20% palmitic acid are $\beta^1$ tending while those with approximately 10% palmitic acid are $\beta$ tending, like sunflower oil which is used in this study. However even though $\beta$ are the most stable, he stated that $\beta^1$ crystals are the most desirable as they are relatively small and can incorporate larger amounts of liquid oil in the crystal network giving the final product a glossy surface and smooth texture.

### 2.2.2 Isolation of microorganisms from water-in-oil emulsions

Fat products are generally of good microbiological quality as the fat acts as a barrier to microbial growth. In high fat emulsions, water is compartmentalized in small droplets. Here, outgrowth of microorganisms is limited and die-off may occur during storage due to a low nutrient supply (van Zijl et al., 2000). On the other hand low fat emulsions have coarser water droplet sizes which are more suitable for microbial proliferation. Ideally, water droplets should be less than 20 µm in diameter in low fat spreads (Bullock et al., 1969). Above this size growth of microorganisms is expected depending on the size and oxygen requirements of the organism.

The most common non microbial spoilage is due to oxidation of fat at the surface and rancidity resulting from fat hydrolysis by naturally occurring lipases or microbial lipases. Microbial spoilage may be due to molds, yeasts or bacteria as discussed below.

Molds belonging to *Penicillium* spp, *Mucor* spp, *Aspergillus* spp cause surface discoloration and off flavours and their growth is promoted by high temperatures (>10°C), free moisture on the product surface and low salt content (van Zijl et al., 2000). Mushtaq et al. (2006) isolated a number of yeasts from butter in Pakistan including *Pichia guilliermondii, Pichia anomala, Candida friedrichii, Debaryomyces vanrijii, Rhodotorula* spp. and many others.
These lipolytic yeasts can grow at low temperatures and low pH levels and may cause yeasty off flavours, changes in texture and a bitter taste. The forementioned spoilage characteristics become manifested when yeasts grow to 6 log CFU/g (Lendenbach et al. 2009; Rose et al. 1993). Aside from fungal spoilage, bacterial spoilage has also been observed in the form of surface taint or putridity by *Pseudomonas putrefaciens*, malty flavor by *Lactococcus lactis* and rancidity due to liberation of free fatty acids by *Pseudomonas fragi* (Jay, 1998).

Pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* spp., *Campylobacter* spp and *E.coli 0157:H7* (ICMSF, 2005) have also been isolated from table spreads. Some of these can survive and even grow at refrigeration temperatures. Caution should be taken during manufacture of table spreads to prevent contamination from surfaces, use of poor quality raw materials and improper heat treatment.

The microbial stability of the spreads is enhanced by addition of preservatives like sorbic acid with levels in European Union of up to 0.1% for fat emulsions (excluding butter) with a fat content of 60% or more and 0.2% for those with fat content less than 60% (EU Council Regulation 1129/2011). The antimicrobial effect of the above preservative is however lowered in the presence of fat in foods (Lund et al., 2000). The oil/water partition coefficient of sorbic acid is 3.3 which means that a large amount of the undissociated sorbate will be found in the fat phase where it is not effective against microbial proliferation. In the presence of solutes such as sucrose and salt, the partition quotient increases to 7 (Sofos et al., 1981). Owing to the above mentioned factors, and the persistence of yeasts in extreme conditions, good manufacturing practices must be applied and low temperatures should also be used in combination with preservatives, acidity, salt concentration etc. As previously mentioned, low fat spreads are more vulnerable to microbiological spoilage than full fat spreads. In such products, the aqueous phase composition become more important for keeping quality.
2.3 EFFECT OF pH, SORBIC ACID AND TEMPERATURE ON MICROBIAL GROWTH

2.3.1 Mechanism of action of pH on microbial growth

pH is one of the factors, along with temperature, dissolved gasses, osmotic pressure, and water activity, that greatly influences microbial growth. It is defined as the measure of the molar concentration of hydrogen ions in the solution. Micro-organisms have minimum, optimum and maximum pH ranges in which they function appropriately. Unfavourable environmental pH can lead to a number of negative effects on the cell such as change of the ionic charges on the molecule, alteration of the cell membrane permeability, decrease of cytoplasm pH and eventually cell death. Unlike bacteria, yeasts and moulds are rather acid tolerant with some growing at pH as low as 1.5 (Devlieghere et al., 2013). The physiological basis of the effect of pH on yeast is not yet completely understood (Deak et al., 2008). It is generally believed that the maintenance of a proton gradient across the plasma membrane against a constant intracellular pH of about 6.5 is vital for a yeast cell for optimal activity of critical metabolic process (Holyoak et al., 1996). Some yeast like Zygosaccharomyces bailii are able to survive harsh acid conditions due to the possession of energy demanding systems that prevent acidification of the cell interior by actively pumping out any acid that penetrates the membrane (Devlieghere et al., 2013).

In order to lower the pH, different techniques (microbial or chemical) have been used in the food industry. The most common microbial technique is lactic acid fermentation which is used in products like yoghurt, dry sausage and sauerkraut. A starter culture with a mixture of micro-organisms is used for lactic acid production which in turn lowers the pH and gives the product its characteristic taste, flavor and texture. Food acidulants such as acetic acid, citric acid, malic acid are usually added in high concentrations, while preservatives like benzoic acid, sorbic acid and proprionic acid are applied at low levels. The above acids are characterized by their dissociation constant, the pK\(_a\) value. The larger the pK\(_a\) value, the smaller the extent of dissociation at any given pH, and the higher the antimicrobial activity. The pK\(_a\) value is therefore an important aspect when choosing preservatives. Other factors
that should be considered include: the product to be preserved, type of spoilage microorganism endemic to it, the pH of the product, the shelf life, and the ease of application (Hui, 2006). Some microorganisms produce acid as they grow which lowers the pH in the surrounding environment and eventually halts growth unless the acid is neutralized. In such cases a buffer can be used to mop up the excess acid and keep the pH constant.

2.3.2 Mechanism of action of sorbic acid and its salts on microbial growth

In general, the increased demand by consumers for lightly processed, safe and convenient foods has stimulated the use of antimicrobial preservatives over the past several years. As previously mentioned, pH is a very important factor but foods become inedible if their pH is outside the range in which microbial growth is possible. The food industry therefore combines pH with preservatives as a form of hurdle technology.

![Molecules of sorbic acid (left) and potassium sorbate (right).](Sigma-Aldrich, 2016)

The molecular weights of sorbic acid and potassium sorbate are 112.13 and 150.22 g/mol respectively. When potassium sorbate is dissolved in water, it ionizes to form sorbic acid which inhibits microbial growth. Potassium sorbate and sorbic acid have a GRAS status and have been recognized for their inhibition of yeasts and molds. The antimicrobial effect of sorbic acid was first used for industrial production in the mid 1950s but was discovered from rowanberry oil in 1859 (Lück et al., 1997). Sorbate affects bacteria to a lesser degree, and some bacteria may even metabolize it. Because sorbates have no effect on microorganisms that produce lactic acid, they are widely used in most cultured dairy products and pickles without interfering with the desired bacterial cure (Hui, 2006). Sorbates are also popular because they exhibit mild organoleptic properties, have a relatively low cost and exhibit a neutral taste in cheeses, baked goods, spreads, margarines, dried fruits, jams etc. The legal
The limit of sorbate expressed as sorbic acid in food is in concentrations in the range of 0.1-0.2% higher levels may cause undesirable changes in the taste of foods (Sofos et al., 2004).

Efficiency of sorbic acid as an inhibitor is based upon the undissociated acid concentration and not the total concentration. Since the cell has a negative charge, only the undissociated molecules can subsequently dissociate in the cell, resulting in an antimicrobial effect. Bell et al., (1958) reported that a drop in pH from 5.3 to 4.3 causes a three-fold increase in the percentage of undissociated sorbic acid. At pH 5.5, the maximum concentration of potassium sorbate that allowed growth was equivalent to 1,493 mg sorbic acid/l, whereas at pH 4.5 the maximum concentration was equivalent to 373 mg sorbic acid/l (Lund et al., 2000). Table 2.2 below clearly illustrates this association between pH and undissociated acid. However, a study done by Eklund (1983) showed that microbial inhibition of sorbic acid may be brought about by both undissociated and dissociated fractions. The microorganisms used in the above mentioned study included Bacillus subtilis, Bacillus cereus, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus and Candida albicans. For most of these microorganisms, the dissociated acid caused more than 50% growth inhibition especially at pH levels greater than 6.

Table 2.2: The percentage of undissociated sorbic acid at different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>3.0</th>
<th>4.0</th>
<th>5.0</th>
<th>6.0</th>
<th>7.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undissociated acid %</td>
<td>97.4</td>
<td>82</td>
<td>30</td>
<td>4.1</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Values obtained from Devlieghere et al., 2013

Besides the percentage of undissociated molecules, solubility is another factor that plays a major role in the use of sorbic acid as a preservative. The salts of sorbic acid i.e. potassium, sodium and calcium sorbate are more soluble than the acid and are therefore the primary form used in food (Ruiz-Capillas et al., 2016). Potassium sorbate exhibits a water solubility of over 50% (58.12 g/100 mL) while sorbic acid is only 0.15% (0.15 g/100 mL). In addition to this, sorbic acid is less effective in products with a high fat content because of its tendency to partition into the fat phase rather than the aqueous phase (Jureja et al., 2001). Hence most of the preservative is not available for anti-microbial activity.
Although a number of researchers like Acton (2013) and Mortimore et al., (1994) have indicated that the exact mechanism of sorbic acid is still unknown, many others have brought up various explanations for this. Melnick et al., (1954) suggested that once inside the cell, the chemical inhibits dehydrogenation of fatty acids, causes membrane damage and disrupts enzyme activity inside the cells.

Jay (1998) reported that the mechanism of action of sorbic acid involves the proton motive force (PMF). Hydrogen ions (protons) and hydroxyl ions are separated by the cytoplasmic membrane with the former, outside giving rise to acidic pH and the latter, inside the cell, giving rise to pH near neutrality. The membrane gradient thus created represents electrochemical potential that the cell employs in the active transport of some compounds for example amino acids. Weak lipophilic acids act as proton-phores. After diffusing across the membranes, the undissociated molecule ionizes inside the cell and lowers intracellular pH. This causes a weakening of the transmembrane gradient such that amino acid transport is affected adversely. Sorbates have also been reported to cause morphological changes in microbial cells including development of yeast cells with dense lipoprotein granules, irregular nuclei, and changes in the genetic material.

### 2.3.3 Effect of temperature on yeast growth

Temperature is a very important factor that influences many reactions within microbial cells. Any microorganism will grow more rapidly as the temperature is increased but until the optimal temperature. Most often yeast cells grow best around the temperature of 30°C (optimal temperature for growth of mesophiles). At 20°C, their growth is greatly reduced and at temperatures of 60°C to 70°C no yeasts are able to grow due to irreversible changes in the plasma membrane and other cell structures (Halasz et al., 1991). At extremely low temperatures (below 10°C), there is a reduced cell number and an extension of the lag phase mainly due to changes that alter the membrane lipid composition. The extent of damage depends on how far the temperatures are below the microorganism’s minimum growth temperature and how long the cells are subjected to those low temperatures. Microscopic studies of yeasts by Malhotra et al., (2008) showed that yeast cells clumped together at low temperatures which limited their growth. It is however important to note that the food and environmental conditions play an important role in the viability of microorganisms at
refrigeration temperatures. Factors such as water activity, pH, and presence of preservatives all have an interactive effect on yeast tolerance to temperature extremes (Rai et al., 2015).

2.4 METHODS USED TO DETERMINE AND QUANTIFY MICROBIAL GROWTH

2.4.1 Spread plate technique

Viable count determinations in this research were done by spreading on agar plates. Spread plates are frequently used for yeast and mold enumeration. The rationale for this is that most yeasts and molds are aerobes and grow best on the surface of a culture medium instead of being placed in its depth. The method involves making ten-fold dilutions and then depositing a measured volume of the sample onto the surface of a pre-poured plate and spreading the liquid uniformly over the agar surface. The assumption is that each viable cell will develop into a single colony (Reynolds, 2011). The bacterial count in the sample is calculated from:

$$X = \frac{A \times V}{I}$$

Where $X$= Number of microorganisms (CFU/ml); $A$= Colony count; $V$= Inverse of level of dilution and $I$= Volume of inoculum added to the petri-dish

The spread plate technique is a very common method for enumeration of microorganisms because it has a number of advantages. Since the plates are always poured and allowed to solidify in advance, there are no concerns about hot agar injury or killing the inoculum (Entiss, 2002). In line with this, there is no condensation forming inside the lids of the petri-dishes, hence there is limited susceptibility to spreaders. Because everything is on the surface, it is much easier to distinguish between a colony and a food particle with spread plates than with pour plates.

However, there exists a few disadvantages of this method. Firstly, the colonies on spread plate have an accurate counting range of 150-200 colonies which is less than the 300 colonies in the pour plate. Therefore depending on the level of contamination, it might prove necessary to run additional dilutions for some samples (Entis, 2002). Plates must be dried just right when taken out of the refrigerator by placing the plates inverted and slightly opened.
under a laminar flow hood. Inadequate drying makes it difficult for the liquid to soak into the agar while excessive drying causes the liquid to be absorbed into the agar so quickly that the inoculum cannot be spread evenly over the surface and also makes a plate more susceptible to drying out during incubation.

Developments in scientific research have come up with ways to automate viable count plating and to a certain extent, reduce the work load and time involved. One example is the use of automated platers such as the spiral plater which will be discussed below.

2.4.2 Spiral plate method for bacterial determination

In 1973, Dr. J. E. Campbell introduced the spiral inoculation method when he launched Model A Spiral Plater. The method determines the number of microorganisms in a solution by the use of a machine which deposits a known volume of sample on a rotating agar plate in an ever decreasing amount in the form of an Archimedes spiral.

![Spiral of Archimedes](image)

**Figure 2.2: The Spiral of Archimedes (IUL-INSTRUMENTS, 1997)**

The amount of sample is controlled and decreased while the dispensing stylus is moved from the centre to the edge of the rotating agar plate. This is achieved through increasing the angle and the radius and also reduction of the sample flow from the center to the outside of the dish. Thus the volume of sample deposited per unit area of plate decreases across the spiral resulting in a dilution effect up to one thousand fold dilution range (IUL-INSTRUMENTS, 1997). On incubation, the colonies develop along the lines where the liquid was originally deposited. A modified counting grid is described which relates area of the plate to volume of the sample. The number of colonies per unit length of line or per unit area on the agar surface is dependent upon the bacterial concentration in the deposited liquid (Gilchrist et al., 1973). The volumes poured along a spiral decrease exponentially so that a single plate hosts several
sample concentrations or dilutions distributed across the spiral. The colonies after incubation are counted with the help of the spiral plate counting grid as will be discussed in a latter chapter.

This method also has its advantages and disadvantages. Among the advantages are: the short preparation time and the speed at which the sample is applied to the plates. The shortened time is due to the fact that fewer dilutions and less material or media is required and also that counting of spiral plates is much faster than counting pour plates. Another advantage is that the Archimedes spiral stops before the meniscus of the agar is reached. Thus colonies are counted only on the clear, level portion of the agar making visual counting easier. The major disadvantage is the high price of the materials and instruments compared to standard plate counts.

2.5 PREDICTIVE MICROBIOLOGY

2.5.1 Principles of predictive microbiology

Predictive microbiology also known as predictive modeling is a distinct discipline in food microbiology which aims at describing microbial behavior with mathematical equations or models. If the microbial behavior (growth, survival, and inactivation) can be described accurately and is reproducible, these models can be used to predict how microorganisms will behave in certain conditions (Devlieghere et al., 2013). This discipline first emerged in the eighties and has since gained popularity especially with its integration with application software. These software programs allow users, even those without interest in mathematics of microbial kinetics, to obtain predictions rapidly, conveniently and cost effectively. Applications of predictive microbiology include techniques used in quality assurance and shelf life prediction in the food industry. Other areas of application are the government, controlling agencies and microbial research in general. To optimize predictive microbiology, a number of models have been developed which include primary, secondary and tertiary models.
2.5.2 Primary models

These models measure the response of the microorganism with time to a single set of conditions. The response can either be direct / indirect measures of microbial population density or products of microbial metabolism (Fakruddin et al., 2011). Examples of primary models are log-linear model, Baranyi model and Gompertz model, a static model which was the most popular until the mid 90s (Baranyi et al., 2004). The log-linear model is used to describe inactivation kinetics as a function of time especially for simple laboratory systems or single strain populations.

It is generally agreed that the most important environmental factor determining growth is the temperature, followed by the pH and water activity, then preservatives, anti-microbial agents and composition of the atmosphere. However while the temperature is controllable during food storage, the other environmental conditions are not since they are changed by the growing bacterial populations. This fact increased the need for dynamic models over the static ones. The Baranyi model became the most widely used primary growth model mainly due to the fact that it can be applied in a fluctuating/ dynamic environment (Baranyi et al., 2004).

![Diagram of microbial growth curve](image)

**Fig 2.3:** Phases and parameters of a typical food microbial growth curve (Baranyi, 2010)

Primary growth curves are often modeled based on sigmoidal curves which have a lag phase, exponential phase, stationary phase and a decay phase. When experiments are done in
duplicate/triplicate, software programs use non-linear regression, specifically non-linear least squares to obtain a best fit which minimizes the distance between the raw data and the fitted data. The growth curve as shown in Figure 2.3 is described by four model parameters: \( Y_o \) (the initial cell count in log CFU/g), \( \lambda \) (duration of the lag phase in hours), \( \mu_{max} \) (maximum growth rate), \( Y_{max} \) (maximum cell count in log CFU/g).

The lag parameter \( \lambda \) represents the time when the intersection of the tangent drawn to the inflexion crosses the initial inoculum level. It is the period when cells adjust to their new environment before growing exponentially. Lag time is greatly dependent on the history and physiological state of the cells. The exponential phase is characterised by the maximum growth rate \( \mu_{max} \). However, the \( \mu_{max} \) is not necessarily the steepest slope of the actual growth curve but expresses a ‘potential’ maximum specific growth rate that is characteristic to the population only if the initial concentration level is low (Baranyi, 2010). Sometimes, the more easily interpreted generation time is used instead of \( \mu_{max} \). Generation time (GT) is the time needed for one cell to double and is calculated by the following expression:

\[
GT = \frac{\log(2)}{\mu_{max}}
\]

The dependence of the parameters of the primary model according to Baranyi is based on the polynomial equation below,

\[
y(t) = y_0 + \mu_{max} \ A(t) - \frac{1}{m} \ln \left\{ 1 + \frac{e^{m\mu_{max}A(t)-1}}{e^{m(y_{max}-y_0)}} \right\}
\]

\[
A(t) = t + \frac{1}{v} \ln \left( \frac{e^{-vt+q_0}}{1+q_0} \right)
\]

where, \( y(t) = \ln x(t), y_0 = \ln x(t_0), m=1, q_0 \) is the initial physiological state of the cells and \( v \) represents the rate of increase of the limiting substrate and is equal to \( \mu_{max} \).

### 2.5.3 Secondary models and tertiary models

Secondary models describe the effect of environmental conditions (temperature, pH, water activity) on primary growth parameters. If a model does not include all the environmental parameters that are paramount in a food, then it is said to be ‘incomplete’ (Baranyi et al.,
Various categorizations of secondary models have been recognised and a few (probabilistic, kinetic, empirical and mechanical) will be discussed here. Probabilistic models predict the chance that microbial growth, or survival or even toxin production will occur (Devlieghere et al., 2013). They are therefore widely used in microbial risk analysis. Kinetic based models are rather complex as they aim at incorporating both secondary and primary models and describing how their interactions affect cell growth or death. An example of this is the Arrhenius model which is described by the Arrhenius equation. Empirical models include polynomial models, square root models, and artificial neural networks. These models simply describe a set of data in a mathematical equation and data fitting but do not consider any underlying phenomena. Mechanical models on the other hand are built based on physical, chemical and biological processes of the microorganism and face a challenge because there is still limited information on various species (McKellar et al., 2003; Devlieghere et al., 2013).

Tertiary models integrate predictive models in user friendly programs and come in software packages such as ComBase, SSSP (Seafood Spoilage and Safety Predictor). ComBase software was used in this research to predict the growth of *Candida guilliermondii* and generate growth curves based on the environmental conditions. Specifically, the Dynamic Modelling Fit (DMFit) tool which is an Excel add-in was used to fit the curves in a sigmoid manner. According to the Institute of Food Research, using DMFit is different from other sigmoid curves like Gompertz and Logistic because it ensures that the mid-phase is very close to linear unlike the pronounced curvature observed in other sigmoid curves. Such software has become very popular among food industries and research organisations who use it to assess microbial risk in foods, and to economically design and produce foods. The software is also used by many individuals to predict thermal and non thermal inactivation of spoilage and pathogenic microorganisms. The SSSP software on the other hand is product specific and is used to predict microbial growth and histamine production in fish and fish products.

**2.5.4 Limitations of predictive microbiology**

Even though this field offers a large number of opportunities, there are some challenges that should be taken into account. Models developed in laboratory broth systems have been
reported to be inappropriate to describe growth on food (Gill et al., 1997). This is due to the variation in temperature and pH at different locations due to the food structure. Aside from the effect of structure, there exists background flora in food which may bring about interactions with the target microorganism. It is also important to note that some reference strains used in modeling do not always exhibit the same characteristics of the product flora.
3 MATERIALS AND METHODS
3.1 MATERIALS

Neutralized bacteriological peptone, bacteriological agar, YNB, sucrose, and NaCl were obtained from Oxoid N.V (Aalst, Belgium). For the phosphate buffer solutions, potassium dihydrogen phosphate (KH$_2$PO$_4$) and disodium hydrogen phosphate (Na$_2$HPO$_4$), were obtained from Chem-Lab NV, Belgium while sodium dihydrogen phosphate (NaH$_2$PO$_4$) was obtained from Sigma-Aldrich, Germany. Other materials from Sigma-Aldrich, Germany were hydrochloric acid (HCl), sodium hydroxide (NaOH) and potassium sorbate. Phosphoric acid (H$_3$PO$_4$) was purchased from Merck, UK. Sunflower oil was bought from Delhaize group, Belgium while the solid fat inES 48 hardstock was obtained from Vandemoortele Lipids N.V.

Aside from the above chemical products, consumables such as pipettes (10 ml), micropipettes (1 ml, 100 µl), petridishes, 0.45 µm sterile membrane filters from Thermo Fisher Scientific, Erembodegem, Belgium were used. Equipment used in the experiment include incubators (22°C, 30°C), refrigerators (4°C, 7°C), Eddy Jet spiral plater (IUL Instruments, Led Techno, Heusden-Zolder, Belgium), vacuum pressure system for membrane filtration, pH meter (Mettler-Toledo, Schwerzenbach, Switzerland), shaker (IKA-WERKE, Staufen, Germany) and the Ultra Turrax (IKA T25 digital, Germany).

3.2 PREPARATION OF MEDIA

3.2.1 Preparation of Peptone Physiological Salt (PPS)

8.5 g of NaCl and 1 g of neutralized peptone were thoroughly mixed with 1000 mL of distilled water and distributed in test tubes which were then autoclaved for 15 minutes at 1 bar and 121°C.

3.2.2 Preparation of Yeast Nitrogen Base Agar (YNA)

6.7 g of Yeast Nitrogen Base media (without amino acids) together with 20 g of sucrose and 20 g of bacteriological agar powder were weighed in an Erlenmeyer flask and thoroughly mixed with 1000 mL of distilled water. The flask and its contents were then heated to boiling with frequent agitation to prevent caramelisation of the sugar at the bottom of the flask. The media was then distributed into 500 mL bottles and autoclaved for 15 minutes at 1 bar and
121°C. The agar was then cooled to 48°C and poured into empty petri-dishes. These were allowed to set and stored under refrigeration temperature.

3.2.3 Preparation of phosphate buffer solutions and Yeast Nitrogen Broth (YNB)

YNB was prepared and diluted with phosphate buffer solutions at different pH levels and before storage the pH was measured and adjusted accordingly. The following paragraphs will contain information pertaining to the preparation of the phosphate buffers according to the European Pharmacopoeia 5.0 (2005) and the preparation of buffered YNB. The pH of the buffer solutions (4.5, 5.5 and 6.5) was adjusted using 6M HCl, or 6M NaOH.

**Phosphate buffer solution pH 3.5:** 68 g of KH$_2$PO$_4$ was diluted with 1000 mL of distilled water and the pH adjusted with 1M H$_3$PO$_4$.

**Phosphate buffer solution pH 4.5:** 0.05 M phosphate buffer solution was prepared by dissolving 6.8 g of KH$_2$PO$_4$ with 1000 mL of distilled water.

**Phosphate buffer pH 5.5:** Solution I was prepared by dissolving 13.61 g of KH$_2$PO$_4$ in 1000 mL of distilled water. Solution II was prepared by dissolving 35.81 g of Na$_2$HPO$_4$ in 1000 mL of distilled water. The final phosphate buffer with pH 5.5 was made by mixing 96.4 mL of solution I and 3.6 mL of solution II.

**Phosphate buffer pH 6.5:** 0.1 M phosphate buffer solution was prepared by dissolving 13.8 g of NaH$_2$PO$_4$ in 900 ml of distilled water.

**Yeast Nitrogen Broth:** 6.7 g of Yeast Nitrogen Base media (without amino acids) together with 20 g of sucrose were weighed and thoroughly mixed with 1000 mL of the corresponding phosphate buffer solution. The mixture was filter sterilized by membrane filtration using 0.45 μm membrane filters and a vacuum pressure system. The sterile media was stored at 4°C for a period of 1 month.

**Yeast Nitrogen Broth with 100 ppm potassium sorbate:** 6.7 g of Yeast Nitrogen Base media (without amino acids), 20 g of sucrose and approximately 134 mg of potassium sorbate were weighed and thoroughly mixed with 1000 mL of the corresponding phosphate buffer solution. The amount of potassium sorbate is as above because on an equivalent
weight basis, the salt has 74% of the activity of sorbic acid, hence higher concentrations are required to obtain the same inhibitory effects (Igoe, 2011). The pH was measured and adjusted if necessary. The mixture was then filter sterilized by membrane filtration using 0.45 μm membrane filters and a vacuum pressure system and stored at 4°C for a period of 1 month.

3.3 PROCEDURE OF CULTURING CANDIDA GUILLIERMONDII

3.3.1 Preparation of culture to be used in the experiments

A fresh strain of Candida guilliermondii was taken from the stock collection at -85°C and cultured in 5 mL of YNB at 30°C for 48 hours. Using a sterile loop, a small amount of the culture was picked and spread over the agar surface using the streak plate technique. In this technique, individual bacterial cells from the original sample become spaced farther and farther apart, and the numbers are reduced in successive sections (Sumbali et al., 2009).

The plates were incubated at 30°C for five days. The characteristic colonies appeared round, smooth, and cream in colour on YNA. A single colony was then picked from the plate and cultivated in 5 ml of YNB for 48 hours at 30°C on a shaker at 200 rpm. Halasz et al., 1991 noted that agitation is important as it increases the amount of dissolved oxygen which in turn increases the level of unsaturated lipids that are essential to the functioning of the cell membrane. Overall this increases the maximum yield as a difference of 1 log CFU/mL is observed between the agitated culture of Candida guilliermondii grown in YNB at 30°C at 200 rpm and the non-agitated culture (Rukundo, 2015).

3.3.2 Experimental set-up

From the fully grown culture which contained 10⁸ CFU/mL, serial dilutions were done in triplicates using the buffered YNB at the different pH levels (3.5, 4.5, 5.5 and 6.5). The serial dilutions were made up to a level of 2 log CFU/mL and these tubes were incubated at 22°C. Plating was done using the spread plate technique every 4 hours for a period of 3 or 4 days and to obtain a minimum of 10 sampling points, and the plates were incubated at 30°C for five days. Another technique that was used especially when higher dilutions were suspected
was the use of the Eddy Jet spiral plater. This same experiment was done but with 100 ppm of potassium sorbate added to the YNB.

![Diagram](image)

**Figure 3.1:** Image summarizing the experimental set up

In addition to the above, similar treatments were done at the different pH levels, with and without potassium sorbate but the tubes were incubated at 7°C. In this case the plating was done every 72 hours for a period of 30 days and the plates were incubated at 30°C and counted after 5 days.

For the experiments with oil, this study aimed at achieving a 40:60 water to oil ratio to represent a three quarter fat spread as defined by EU regulation, EC 1234/2007. 10 mL of YNB with 2 log CFU/mL at the different pH levels (3.5, 4.5, 5.5 and 6.5) with and without 100 ppm potassium sorbate was mixed with 15 mL of sunflower oil and the above experiment was repeated. During sampling, thorough mixing was done with a vortex, in order to avoid sampling errors due to yeast sedimentation, before carefully taking a given aliquot from the water phase. To ensure that the ratio of water to oil remained constant, then same amount/aliquot was taken from the oil phase during each sampling point. The oil experiments were also performed at 22°C and 7°C.
For the solid fat experiments, an equal amount of sunflower oil and melted inES 48 hardstock was mixed with an Ultra-Turrax homogenizing machine. The mixture was cooled down to 37°C to prevent damage to the yeast present in the inoculum. 15 mL of the fat mixture was then distributed into 10 mL of YNB with 2 log CFU/mL at the different pH levels (4.5, 5.5 and 6.5) with and without 100 ppm potassium sorbate. Different samples were made according to the number of samplings planned. During sampling, a hole was drilled into the solid fat to access the YNB. Through mixing was done with a vortex before taking off the required amount of inoculum for plating. The plates were incubated at 30°C for 5 days and the experiments were performed at 22°C and 7°C. An image showing the different matrices used in the experiment is shown in Figure 1 of the Annex.

### 3.3.3 Enumeration methods

The YNA plates were incubated and the colonies were counted after 5 days. The colony count of the plates which were inoculated by spread plate technique was calculated from the formula:

\[
X = \frac{A \cdot V}{I}
\]

where \(X\) = number of microorganisms (CFU/mL); \(A\) = colony count; \(V\) = level of dilution; and \(I\) = amount of inoculum in mL added to the plate.

However the colony count for the plates which were inoculated using the Eddy Jet spiral plater was different. A counting grid as shown below was used.

![Spiral plating counting grid](image)

**Figure 3.3:** Spiral plating counting grid (IUL-INSTRUMENTS, 1997)
The counting grid is divided into 8 equal wedges and each wedge is divided by 4 arcs with other lines within these arcs for ease of counting (Maturin et al., 2001). Any of the wedges was chosen and the colonies counted from the outer edge of the first segment (shaded zones) towards the center until 20 colonies had been counted. If there were any remaining colonies in the segment in which the 20\textsuperscript{th} colony occurred, they were also counted. Once a segment was counted, the opposite one was counted too and the formula below was applied to obtain the CFU/ml.

\[
\text{Count (CFU/ml)} = \frac{\text{Sum of colonies in area}}{\text{Volume (mL) for grid area}}
\]

The volume that was represented by various parts of the counting grid is shown in the operator’s manual that accompanies the machine while the sum of the colonies was got from addition of colonies in opposite segments. In cases where 20 CFU were not within the 4 segments of the wedge, the entire plate was counted (JOINT FAO, 2006). If the number of colonies in the 2\textsuperscript{nd}, 3\textsuperscript{rd}, or 4\textsuperscript{th} segment, which contained the 20\textsuperscript{th} colony exceeded 75, the plate was recounted by counting the circumferentially adjacent segments in all 8 wedges, the minimum number of colonies should be 50 (FDA, 2015). If plates showed no colonies, then 0 was recorded and if there were excessive colonies then a record of TNTC (Too Numerous to Count) for the largest dilution factor was recorded.

The results from the above methods were log transformed and using the Combined Database (ComBase) software, modeled according to the Baranyi-Roberts model. The length of the lag phase in hours, the initial and final log CFU/mL, and the maximum growth rate were obtained. The maximum growth rate was then transformed into generation time (GT) using the formula;

\[
GT(\text{hrs}) = \frac{\log_{10}(2)}{\mu_{\text{max}}}
\]
3.3.4 Statistical Analysis

R software version R i386 3.2.2 was used to compare maximum growth rates and generation times among the different factors involved in this study and a 5% significance level was applied for all statistical tests. A one way ANOVA was done to compare the means of groups with one factor and a two way ANOVA was done for cases where two independent variables/factors had to be compared. Boxplots and QQ plots were used for basic data exploration. To test for normality and equality of variance, the Kolmogorov Smirnov test and Levene test were used respectively. However, owing to the fact that each sampling from the solid fat experiments was from a different/ independent recipient, comparison between groups was done using the overlap of intervals.
4 RESULTS AND DISCUSSION
4.1 STRUCTURAL CHARACTERISTICS OF *Candida guilliermondii*

![Images of Candida guilliermondii colonies in the absence of potassium sorbate](left) and in the presence of potassium sorbate (right)

*Figure 4.1*: Images of *Candida guilliermondii* colonies in the absence of potassium sorbate (left) and in the presence of potassium sorbate (right)

As shown above in *Figure 4.1*, a clear difference can be seen between the colonies from the experiments where potassium sorbate was used (right) and those without addition of sorbate (left). *Candida guilliermondii* colonies are off white in colour for both cases. However in the presence of sorbate, the edges of the colonies are irregular and rough compared to what is observed as smooth colonies in its absence. Many studies on bacterial and yeast cells have reported changes in cell morphology and appearance when exposed to sorbate but the exact mechanism by which this happens is still not known (Mendonca, 1992). Proposed explanations are related to the ability of sorbate to obstruct several reactions needed to form the cell wall and membrane. Alimkhamedova (1977) reported that at concentrations as low as 0.025 to 0.05% of sorbic acid yeast cells will develop irregular nuclei, mitochondria of different sizes and increase in density of vacuoles and phospholipoprotein granules. The combination of all the above could lead to an overall disfigurement of the cell.
4.2 EFFECT OF pH ON THE GROWTH OF *CANDIDA GUILLIERMONDII* IN DIFFERENT MATRICES AT 22°C.

4.2.1 Effect of pH on the growth of *Candida guilliermondii* in a matrix of YNB versus YNB + sunflower oil

![Growth curves of *Candida guilliermondii*](image)

**Figure 4.2.** Growth curves of *Candida guilliermondii* incubated in matrices of YNB (▬) and YNB + sunflower oil (■■■) at 22°C at different pH levels as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB represented by (●), and (▲) for YNB + sunflower oil. R-square > 95% for all fittings.

For the experiments with only YNB, the yeast inoculated at pH 3.5 exhibited the slowest growth among all the pH levels while the other pH levels had an almost similar growth pattern. This was confirmed by the statistical test which showed significant differences.
between generation time and lag time at pH 3.5 and the other pH levels (p < 0.05). There were no significant differences between the generation times at pH’s 4.5, 5.5 and 6.5. A summary of all the generation and lag times is given in Table 1 and 2 of the Annex. Walker (1998) reported that although yeasts are capable of growth over a wide pH range (2-8), their optimum pH for growth exists between 4 and 6, which was confirmed for Candida guilliermondii in this study. Studies by Tokuokak (1993) and Narendranath et al. (2007) on Zygosaccharomyces rouxii and Saccharomyces cerevisiae respectively also revealed growth over wide pH levels and showed a similar tolerance for low pH by these species. Mechanisms of yeast adaptation to low pH mainly involve energy demanding systems that enable plasma membrane transporters to pump out the acid that penetrates into the cell (Devlieghere et al., 2013; Mira et al., 2014). Aside from the previously mentioned mechanism, some studies have shown that resistance to low pH may be connected to changes in the membrane conductivity to H+ or possession of special gene expressions like those that code for protein kinase C in Sacchromyces cerevisiae, which is required for cell integrity (Fernandes et al., 2006). Some yeasts also have cell wall sensors and different pathways that are activated in response to acid stress (Brandao et al., 2014).

With addition of oil, the effect of pH was not greatly influential as differences in generation time at all the pH levels were not as prominent as observed with the samples with only YNB. In general, the low pH levels (3.5 and 4.5) had slightly lower growth rates than the higher ones (5.5 and 6.5) but shorter lag times. Evidence from studies about the effect of fats and oils on bacteria by Lansberg (1920) and Lichtenstein (1939) showed decreased bacterial activity as these components caused a lack of oxygen and water. However there has since been several contradictions among available literature. In order to make a more feasible and practical comparison between the samples with sunflower oil and those without, a critical spoilage level was set at 6 log CFU/ml as seen in Figure 4.2. At pH 3.5, 5.5 and 6.5, the spoilage level is reached faster in the presence of oil than in its absence while at pH 4.5, the samples are identical. Prachaiyo et al, 2003 indicated that growth in oil/water emulsions is strongly dependent on the composition of the aqueous phase in terms of nutrients, pH, presence of preservatives and the physical structure of the emulsion. Microorganisms in general grow and multiply in the aqueous phase hence the existence of the aqueous phase in form of droplets will ensure lower growth rates due to limitations in nutrient supply and
space (Brocklehurst et al., 1994; Kumar et al., 2008). This was not observed in this experiment as the aqueous phase was fully available to the yeast since the matrix was not emulsified and a small interphase existed between the sunflower oil and the YNB.

Table 4.1. Lag time (hours) of Candida guilliermondii at different pH levels

<table>
<thead>
<tr>
<th>pH</th>
<th>3.5</th>
<th>4.5</th>
<th>5.5</th>
<th>6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (hours): YNB</td>
<td>10.36 ±3.00</td>
<td>2.84±0.73</td>
<td>2.92±0.8</td>
<td>3.06±1.07</td>
</tr>
<tr>
<td>Lag time (hours): YNB + sunflower oil</td>
<td>2.74±1.91</td>
<td>3.06±2.25</td>
<td>5.65±2.55</td>
<td>5.33±1.1</td>
</tr>
</tbody>
</table>

Mean lag times shown ± standard deviation

Many authors have studied the effect of pre-incubation conditions on the duration of the lag phase. Both Membré et al., 1999 and Francios et al., 2005 reported reduced lag times when Listeria monocytogenes was cultured in conditions (temperature and pH) similar to those of pre-incubation. In this experiment Candida guilliermondii was pre-cultured in unbuffered YNB at pH 5.4 ± 0.2 before it was transferred and grown at the above pH levels. However the lag time at pH 5.5 was not significantly different (p > 0.05) from that at pH 4.5 and 6.5. The same effect was observed in the presence of oil. This could be explained by the ability of yeasts to quickly grow and adjust to conditions over a wide range of pH levels.

4.2.2 Effect of pH on the growth of Candida guilliermondii in a matrix of YNB versus YNB + sunflower oil + inES 48 hardstock

The experiments with solid fat were only performed at pH 4.5, 5.5 and 6.5. This is because the purpose of this study was to quantify growth parameters (lag phase and maximum growth rate) of Candida guilliermondii. It was observed that with addition of potassium sorbate at pH 3.5 complete inhibition occurred. It was therefore decided to omit this condition.

With solid fat addition to YNB, there were minor differences in growth among the different pH levels. However, higher growth rates were realised in comparison to those with only YNB (Figure 4.3). With higher growth rates at all pH levels, the critical spoilage level was reached faster in the presence of solid fat compared to the samples with only broth. This observation could be explained by the fact that the majority of C. guilliermondii are lipase
positive (Zullo et al, 2010). Through hydrolysis of the triglycerides, the by-products of this breakdown could be used in the metabolism of the yeast hence facilitating faster growth in the presence of oil or fat.

![Figure 4.3](image)

**Figure 4.3.** Growth curves of *Candida guilliermondii* incubated in matrices of YNB (▬) and YNB + sunflower oil + INES48 hardstock (∙∙∙) at 22°C as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB represented by(●) and (■) for YNB + sunflower oil + inES 48 hardstock. **R-square > 95% for all fittings**

4.2.3 Effect of pH on the growth of *Candida guilliermondii* in a matrix of YNB + sunflower oil versus YNB + sunflower oil + inES 48 hardstock

As presented in **Figure 4.4**, higher growth rates were observed with solid fat than liquid oil.
**Figure 4.4.** Growth curves of *Candida guilliermondii* incubated in matrices of YNB+sunflower oil (⋯) and YNB + sunflower oil + INES48 hardstock (⋯) at 22°C as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB + sunflower oil represented by (▲) and (■) for YNB + sunflower oil + inES 48 hardstock. R-square > 95% for all fittings.

The lagtimes in these solid fat experiments were the shortest compared to those in the matrix of broth or liquid oil. In addition to the previously mentioned lipolytic nature of this yeast, this study suggests that *C. guilliermondii* may have special interest in metabolism of saturated lipids compared to unsaturated ones. This assumption is yet to be confirmed by running a lipase assay for this yeast. Wiseman (1984) revealed that variations in the kind of fatty acids also affects microbial growth. Sunflower oil contains approximately 70% linoleic acid, 20% oleic acid and 0.6% triolein (Martinez-Force *et al.*, 2015; Gunstone, 2011).
Laboratory trials done on several yeast strains including *C. guilliermondi* in extra virgin olive oil showed increased inhibitory effect by oil components like linoleic acid, oleic acid, and triolein (Zullo *et al.*, 2010). Furthermore, Morton *et al.*, 1938 reported that any oxidation product may be harmful to microorganisms. The degree of fat unsaturation therefore may also have an impact on the yeast growth, as the higher the unsaturation the higher the probability of oxidation.

### 4.3 EFFECT OF POTASSIUM SORBATE ON THE GROWTH OF *CANDIDA GUILLIERMONDII* IN DIFFERENT MATRICES AT 22°C.

#### 4.3.1 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a matrix of only YNB.

![Boxplots showing the maximum growth rates of groups without sorbate (1-4) and those with sorbate (5-8) as obtained by plate counts. Assumptions of normality and equal variance fulfilled (p >0.05).](image)

**Figure 4.5.** Boxplots showing the maximum growth rates of groups without sorbate (1-4) and those with sorbate (5-8) as obtained by plate counts. Assumptions of normality and equal variance fulfilled (p >0.05).

Boxplots in **Figure 4.5** were drawn for a quick data exploration of the inhibitory effect of sorbate. The data was grouped as follows: group 1 (pH 3.5:0 ppm sorbate), group 2 (pH
4.5:0ppm sorbate), group 3 (pH 5.5:0ppm sorbate), group 4 (pH 6.5:0ppm sorbate), group 5 (pH 3.5:100ppm sorbate), group 6 (pH 4.5:100ppm sorbate), group 7 (pH 5.5:100ppm sorbate) and group 8 (pH 6.5:100ppm sorbate). It was immediately clear that the groups without sorbate (groups 1-4) together with groups 7 and 8 had higher maximum growth rates compared to some groups with sorbate (group 5 and 6). This emphasizes the pH dependence of the activity of sorbate because growth rates of *Candida guilliermondii* incubated in buffered YNB at higher pH levels with 100ppm of sorbate do not differ from the groups where no sorbate was added. To confirm this, a two way ANOVA was done to show that the effects of pH and sorbic acid were not merely additive but that the effect of one may depend on the level of the other one. A model with an interaction effect was tested with the following hypotheses:

Ho: There is no interaction between pH and sorbic acid

H1: There is an interaction between pH and sorbic acid

A saturated model was fitted which fulfilled the assumptions of normality and homoscedasticity. The output showed a highly significant interaction effect (p < 0.05). This confirmed the concept that the effect of potassium sorbate is highly dependent on the pH level. The effect of potassium sorbate is further illustrated in Table 4.2 below where a comparison between the groups with sorbate and those without is given for the lag and generation times.

**Table 4.2. Lag and generation times (hours) of *Candida guilliermondii* in the presence and absence of sorbate at varying pH levels**

<table>
<thead>
<tr>
<th></th>
<th>Lag time (Hours)</th>
<th>Generation time (Hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without sorbate</td>
<td>With sorbate</td>
</tr>
<tr>
<td>pH 3.5</td>
<td>10.36±3.0</td>
<td>∞</td>
</tr>
<tr>
<td>pH 4.5</td>
<td>2.84±0.73</td>
<td>∞</td>
</tr>
<tr>
<td>pH 5.5</td>
<td>2.92±0.80</td>
<td>6.13 ± 1.33</td>
</tr>
<tr>
<td>pH 6.5</td>
<td>3.06±1.07</td>
<td>2.44 ± 1.60</td>
</tr>
</tbody>
</table>

Means obtained from three replicates and ± denotes standard deviation; within a row, means of lagtime / generation time with different letters are significantly different at α = 0.05; ∞ means an extended lag phase; - means no generation time.
With sorbate addition, the lagtimes at pH’s 3.5, 4.5 and 5.5 were significantly different (p <0.05) from the samples without sorbate. There was no effect of sorbic acid on the lag time at pH 6.5. Concerning the generation time, no differences were observed between the samples with or without sorbate at higher pH values (5.5 and 6.5) unlike the lower ones (3.5 and 4.5) where sorbate exhibited efficient inhibitory activity. This increased antimicrobial activity at the lower pH levels is due to the fact that they are closer to the dissociation constant of sorbate (pKₐ = 4.76). As the pH approaches this pKₐ value, there is an increased amount of undissociated acid which is said to be the effective antimicrobial form. However some studies have shown effectiveness of sorbate at pH levels of 6.5 and 7.0 (Eklund,1983,Sofos et al.,1981) due to inhibition caused by the dissociated sorbic acid. At such high pH levels, the effect of the dissociated acid is 10-600 times less than that of the undissociated acid (Eklund,1983). Contrary to these studies, there was no observable effect of sorbate at pH 6.5 in this experiment.

Naidu(2000) noted that sorbate has a bigger influence on extension of the lag phase than on the rate of growth in bacterial inhibition. In this study both the lag phase and exponential phase were affected at the low pH levels. However at pH 5.5, the above statement was confirmed as significant differences were only observed with the lag times but sorbate did not have an effect on the generation time.

This anti-microbial effect of sorbate has been related to changes in the integrity, functionality and porosity of the cell membrane which in turn affects substrate uptake, toxin release, and electron transport (Reinhard et al., 1981; Statham et al., 1988; Sofos, 1989). Potassium sorbate has also been known to interfere with fatty acid metabolism in the cell (Hui, 2006). This effect is dependent on the type of microorganism and the species/strain, level of sorbic acid, and environmental factors like pH of the food, water activity, temperature, and other additives among others. Some yeasts belonging to the genera Zygosaccharomyces, Candida, Pichia and Saccharomyces have been shown to develop resistance to the inhibition by sorbate. A study by Lenovich et al., 1988 showed that cells that were previously incubated in media with sorbate and glucose or sucrose developed sorbate resistance when subsequently exposed to sorbate conditions. This mechanism of resistance especially in environments with low water activities can be explained by a decrease in membrane pore size.
which makes it difficult for the sorbate to flow into the cell (Naidu, 2000). In this particular experimental design, pre-incubation conditions only comprised of YNB therefore no resistance to sorbic acid inhibition was expected.

4.3.2 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a system with YNB + sunflower oil

![Figure 4.6: Generation times of *Candida guilliermondii* at different pH levels with and without sorbate addition as obtained by plate counts; means obtained from three replicates; error bars represent standard deviation.](image)

The generation times of *Candida guilliermondii* in a system with sunflower oil and sorbate were generally higher than in a matrix with only oil at all the pH levels. Generation time at pH 3.5 with potassium sorbate was significantly different from the rest of the pH levels with sorbate (*p* <0.05) which illustrates the strong inhibitory effect of sorbate at this low pH. In addition to this, no significant differences were observed between the generation time of the samples with and without sorbate at pH 4.5, 5.5 and 6.5.

4.3.3 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a system with YNB versus YNB + sunflower oil

A comparison was also made between the system that had potassium sorbate without oil (YNB only) and the one with YNB + sunflower oil and sorbate as shown in Figure 4.7.
Figure 4.7. Growth curves of *Candida guilliermondii* incubated in matrices of YNB (---) and YNB + sunflower oil (---) with potassium sorbate addition at 22°C as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB represented by (●), and (▲) for YNB + sunflower oil. R-square > 95% for all fittings.

The curves above show a clear difference between the low pH levels and the high ones with regards to spoilage. While the yeast at pH 5.5 and 6.5 makes it to the critical spoilage level after 40-60 hours in both the broth and the oil matrix, it only attains this level after 70 hours at pH 4.5 in the oil matrix. This level is not reached in either matrices at pH 3.5 and in the broth matrix at pH 4.5.
In the case of generation time, there was complete inhibition of the yeast at pH 3.5 and 4.5 in YNB while YNB + oil had growth at the mentioned pH levels. This illustrates the phenomenon of partitioning of the sorbic acid between the aqueous phase and the oil phase. Gaonkar et al., (1995) suggested that carboxylic acid preservatives like sorbic acid and benzoic acid tend to associate with the oil phase and this reduces the activity of the preservative in the aqueous phase where microbes are known to proliferate. When only the aqueous phase is present like in the matrix of only YNB, no partitioning occurs and the sorbic acid is solely present to exert its antimicrobial effects by accumulating within the cells and reducing the pH within the cell or reacting with metabolic enzymes that have important sulfhydryl groups that are required for growth. The distribution coefficient of sorbic acid is the ratio of the concentration of undissociated sorbic acid in the lipid phase to its concentration in the aqueous phase. The undissociated sorbic acid has an oil/water partition coefficient of 3.3 which means a large proportion of it will be dissolved in the fat phase (Lund et al., 2000). The partitioning of sorbic acid is dependent on the type and amount of fat, pH, surface interfacial area, droplet size and other ingredients (Denyer et al., 2007). The antimicrobial effect of sorbic acid is reduced as the level of fat is increased, and the reduction effect is highest in acidic foods. According to Lund et al., 2007, irrespective of the amount of oil in the system, at pH 5.0, the same concentration of undissociated sorbic acid remains in the water phase. However, as the pH reduces below 5.08, any increase in the ratio of oil to water causes a reduction in the concentration of undissociated sorbic acid in the water phase. In the food industry, it is necessary to know how the preservative partitions between the oil and water phases since the amount that dissolves in the lipid is a “loss” (Cheng et al., 2010).

**4.3.4 Effect of potassium sorbate on the growth of Candida guilliermondii in a system with YNB + sunflower oil + inES 48 hardstock**

Higher generation times were observed when sorbate was added to the solid fat matrix at pH levels 4.5 and 6.5 (Figure 4.8). A similar observation was obtained for the lag time at the aforementioned pH levels. At pH 5.5 however, sorbate action had a bigger influence on the lag time, which was greatly extended (33 hours) compared to the other pH’s than on the generation time. This observation implies that at pH 5.5 in a matrix of YNB+sunflower oil+
inES 48 hardstock, *Candida guilliermondii* multiplies faster in the presence of sorbate than in its absence. This condition will be re-done to confirm this finding.

**Figure 4.8:** Generation and lag times of *Candida guilliermondii* at different pH levels with and without sorbate addition as obtained by plate counts; means obtained from three replicates; error bars represent standard deviation.

### 4.3.5 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a system with YNB+ sunflower oil versus one with YNB + sunflower oil+ inES 48 hardstock

As seen in **Figure 4.9**, the systems with solid fat all had shorter generation times with sorbate than those with liquid fat. This indicates that the level of sorbic acid in the aqueous phase is less in the presence of solid fat than liquid oil.

Due to the different solubility of sorbic acid in different fats, its partition coefficient also depends on the type of fat (Luck *et al.*, 1997). There is limited literature available on the partitioning of preservatives in fat products that are made out of blends with both solid and liquid fat. The evaluation of how preservatives partition in oil in water emulsions is also difficult as during use their structure may be damaged causing serum leakage in the remaining product. Heinz (1991) noted that partitioning of sorbic acid in the presence of crystallized fat is low due to the inertness of solid fat. The opposite was however observed in
these experiments. This is explained by the presence of an appreciable amount of sunflower oil in the solid fat matrix. In addition to this, a certain percentage of the inES 48 hardstock is in liquid form at 22°C. An experiment is scheduled for the measurement of the Solid Fat Index of this hardstock in order to know the exact proportions of solid and liquid phases. As a result, the undissociated sorbic acid partitioned into the oil phase and was thus unavailable to suppress the proliferation of the yeast in the aqueous phase.

**Generation time (22°C)**

![Generation time graph](image)

**Figure 4.9.** Generation times of *Candida guilliermondii* incubated in matrices of YNB + sunflower oil (Liquid oil) and YNB + sunflower oil+ inES 48 hardstock (Solid fat) with potassium sorbate addition at 22°C as obtained by plate counts.
4.4 EFFECT OF pH ON THE GROWTH OF *CANDIDA GUILLIERMONDII* IN DIFFERENT MATRICES AT 7°C.

4.4.1 Effect of pH on the growth of *Candida guilliermondii* in a matrix of only YNB

Table 4.3. Generation time in hours of *Candida guilliermondii* grown in YNB media at 22°C and 7°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Generation time (hrs): 22°C</th>
<th>Generation time (days): 7°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>6.00±1.49</td>
<td>2.67±0.95</td>
</tr>
<tr>
<td>4.5</td>
<td>3.63±0.06</td>
<td>0.66±0.06</td>
</tr>
<tr>
<td>5.5</td>
<td>3.47±0.03</td>
<td>0.77±0.23</td>
</tr>
<tr>
<td>6.5</td>
<td>3.51±0.06</td>
<td>0.58±0.04</td>
</tr>
</tbody>
</table>

Mean generation times obtained from three replicates and ± denotes standard deviation

In general, *Candida guilliermondii* multiplied faster at 22°C than at 7°C. Temperature has an effect on all aspects of microbial growth, metabolism and viability. The optimum temperature for growth of yeasts is 30°C and their growth is greatly reduced at 20°C (Halasz *et al.*, 1991). Any temperature increase above 20°C will lead to a more rapid growth and a higher specific growth rate. At low temperatures membranes become viscous and their fluidity decreases. If temperature is further lowered, the membrane undergoes a phase change to a gel/solid making it difficult to obtain substrates from their environment (Nedwell, 1998).

4.4.2 Effect of pH on the growth of *Candida guilliermondii* in a matrix of YNB versus YNB + sunflower oil

As shown by the lag times in Table 4.4 when in a matrix with sunflower oil, the *C.guilliermondii* cells adjusted faster to the cold environment than in the absence of oil. However, when it came to the exponential phase, in general the generation times of the YNB samples were slightly lower than those with sunflower oil. And as a consequence, the *Candida guilliermondii* in the YNB samples reached the stationary phase faster than the YNB + sunflower oil samples. This is opposite to what was observed at 22°C, where the samples of YNB versus YNB + sunflower oil showed no major differences in growth rate.
especially at pH levels 4.5, 5.5 and 6.5. This emphasizes the importance of low temperature storage for matrices of this kind. Another important aspect is low pH because at 7°C, both matrices at pH 3.5 exhibited the longest time to get to stationary phase. By the end of the experiment, *C. guilliermondii* in YNB had not reached the stationary phase.

**Table 4.4. Lagtimes (days) and days till the stationary phase in YNB versus YNB + sunflower oil at 7°C.**

<table>
<thead>
<tr>
<th></th>
<th>pH 3.5</th>
<th>pH 4.5</th>
<th>pH 5.5</th>
<th>pH 6.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lagtime (days): YNB</td>
<td>3.78 ± 3.97</td>
<td>3.20 ± 0.51</td>
<td>2.72 ± 1.03</td>
<td>3.72 ± 0.56</td>
</tr>
<tr>
<td>Lagtime (days): YNB+ oil</td>
<td>0.69 ± 0.54</td>
<td>1.42 ± 0.49</td>
<td>0.81 ± 0.24</td>
<td>-</td>
</tr>
<tr>
<td>Days till stationary phase: YNB</td>
<td>∞</td>
<td>16.6</td>
<td>17.9</td>
<td>14.7</td>
</tr>
<tr>
<td>Days till stationary phase: YNB+oil</td>
<td>24.9</td>
<td>24.3</td>
<td>23.01</td>
<td>23.01</td>
</tr>
</tbody>
</table>

Means obtained from three replicates and ± denotes standard deviation; ∞ means that the stationary phase was not reached by the end of the experiment.

4.4.3 Effect of pH on the growth of *Candida guilliermondii* in YNB, YNB + sunflower oil and YNB + sunflower oil + inES 48 hardstock

As previously observed at 22°C, the samples with solid fat in their matrix, also had higher growth rates than those with only the broth at 7°C (*Figure 4.10*). This same result was seen when the growth of *Candida guilliermondii* in YNB + sunflower oil was compared to that in YNB + sunflower oil + inES 48 hardstock (*Figure 4.11*). The presence of solid fat could have influenced a shortened lag time at all pH levels when compared to the samples with only broth but this was not the case when compared to the samples with broth and oil. As a result of the above, the critical spoilage level of 6 log CFU / mL was reached first with the solid fat samples at all the pH levels. In addition to these, there were no differences observed among the pH levels. These findings support the earlier mentioned assumption of utilisation of saturated fat components by *Candida guilliermondii* which is yet to be confirmed by a lipase essay.
Figure 4.10. Growth curves of *Candida guilliermondii* incubated in matrices of YNB(■) and YNB + sunflower oil + inES 48 hardstock (∙∙∙) at 7°C as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB represented by (●), and (■) for YNB + sunflower oil + inES 48 hardstock. R-square > 95% for all fittings.
Figure 4.11. Growth curves of *Candida guilliermondii* incubated in matrices of YNB + sunflower oil (---) and YNB + sunflower oil + inES 48 hardstock (…) at 7°C as obtained by plate counts; the curves are constructed from three replicates. Sampling points for YNB + sunflower oil represented by (▲), and (■) for YNB + sunflower oil + inES 48 hardstock. R-square > 95% for all fittings.
4.5 EFFECT OF POTASSIUM SORBATE ON THE GROWTH OF *CANDIDA GUILLIERMONDII* IN DIFFERENT MATRICES AT 7°C.

4.5.1 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a matrix of only YNB

Table 4.5. Generation time in hours of *Candida guilliermondii* grown in YNB media with and without potassium sorbate at 7°C.

<table>
<thead>
<tr>
<th>pH</th>
<th>Generation time (days) Without sorbate (7°C)</th>
<th>Generation time (days) With sorbate (7°C)</th>
<th>Generation time (hours) With sorbate (22°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>2.67±0.95</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>4.5</td>
<td>0.66±0.06</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>5.5</td>
<td>0.77±0.23</td>
<td>1.05±0.02</td>
<td>3.10±0.07</td>
</tr>
<tr>
<td>6.5</td>
<td>0.58±0.04</td>
<td>1.05±0.01</td>
<td>3.05±0.18</td>
</tr>
</tbody>
</table>

Means of generation times obtained from three replicates and ± denotes standard deviation

At 7°C, there were significant differences between the generation times of samples with sorbate and those without (p value < 0.05) for pH levels 3.5, 4.5 and 6.5. At pH 5.5 there was no significant difference showing that the sorbate did not have an effect on the growth rate of the yeast. The preservative had a prominent effect on the lag time at pH 5.5 than on the growth rate. The sorbate was most effective at the low pH levels which are very close to its pKₐ value. As shown in Table 4.5, inhibition of microbial growth by sorbic acid was found to be more effective as storage temperature decreased (Tuncan *et al.*, 1985). This could be because sorbates in aqueous food systems, are unstable and degrade through oxidation to form carbonyl compounds like acrolein, formic and malonic acid which have minor preservative effects compared to sorbic acid (Thakur *et al.*, 1994). This sorbic acid degradation has been linked to increased temperatures and is less common at low temperatures.
4.5.2 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a system with YNB versus YNB + sunflower oil

![Graph showing generation times at different pH levels](image)

**Figure 4.12.** Generation times of *Candida guilliermondii* at different pH levels in systems with and without oil in the presence or absence of potassium sorbate at 7°C as obtained by plate counts; means obtained from three replicates; XX means no growth.

In general, the generation and lag times at 7°C were higher than those at 22°C. In the presence of YNB and sunflower oil, *Candida guilliermondii* grown at pH 3.5 showed the highest generation time followed by 4.5, 6.5 and 5.5 respectively. A comparison was made between the samples with YNB + sunflower oil and those with the same matrix but with sorbate addition at 7°C. There were no major differences observed between the generation times of the above samples at pH 4.5, 5.5 and 6.5 (p > 0.05). However, there were differences between samples at pH 3.5 (p value<0.05). The presence of 100 ppm sorbate at this low pH and a low temperature caused complete inhibition of *Candida guilliermondii*. The partitioning of potassium sorbate was also observed when the samples with broth and sorbate were compared with samples with oil and sorbate. Complete inhibition was observed at pH 3.5 and 4.5 with YNB and sorbate but with oil, it was only observed at pH 3.5. In the oil
matrix, more significant differences due to sorbate where observed with the lag times than the generation times at the different pH levels.

4.5.3 Effect of potassium sorbate on the growth of *Candida guilliermondii* in a system with YNB + sunflower oil + inES 48 hardstock

Figure 4.13. Generation times of *Candida guilliermondii* incubated in matrices of YNB, YNB + sunflower oil (Oil) and YNB + sunflower oil+ inES 48 hardstock (Solid fat) with potassium sorbate addition at 7°C as obtained by plate counts.

There was no significant difference of the effect of sorbate in the three matrices at pH 5.5 and 6.5. This was expected as sorbate is most active at lower pH levels like pH 4.5 where major differences were shown in Figure 4.13 above. At this pH, there was complete growth inhibition of *Candida guilliermondii* in the matrix of YNB only and that of YNB + sunflower oil + inES 48 hardstock. This is because of the high concentration of sorbic acid in the aqueous solution in both systems. As mentioned in section 4.3.5, Heinz (1991) reported that the partitioning of a preservative between the fat and water phases remains the same for vegetable oils, but is lowered when fat becomes crystallized. This explains the observations above as the solid fat matrix despite containing an equal amount of sunflower oil, was crystalline at 7°C. The crystalline structure does not favour partitioning of sorbic acid which is more inclined to dissolve in a liquid network.
5 CONCLUSION AND FUTURE PERSPECTIVES
5.1 CONCLUSION

In summary, pH played an important role in the growth of *Candida guilliermondii* especially in a matrix of only broth. When sunflower oil or a combination of sunflower oil and inES 48 hardstock (solid fat) were incorporated in the broth matrix, no major differences in generation times were observed among the different pH levels. The shortest generation times were observed in matrices that contained solid fat, which prompted the assumption that *Candida guilliermondii* utilizes various components of saturated fat for its metabolism. Many studies have discussed that Good Manufacturing Practice alone cannot be relied upon to stop yeast growth in case of contamination, hence the importance of preservatives like sorbic acid and its salts. The action of sorbic acid is however pH dependent and was shown to be less effective at pH 5.5 and 6.5 compared to pH 3.5 and 4.5 where complete growth inhibition was observed. In the presence of oil or solid fat, there was partitioning of the undissociated sorbic acid into the lipid phase leading to decreased levels of undissociated acid in the aqueous phase and as a result yeast growth occurred at pH 3.5 and 4.5. The influence of storage at low temperature was also studied and the results showed significant differences between the generation and lag times at 22°C and 7°C. The combination of low temperature, sorbate, and low pH lead to complete growth inhibition at pH 3.5 which did not occur at 22°C. Due to the crystallization of fat at 7°C, the solid fat matrix became inert with regards to the partitioning of the acid, hence no growth was observed at pH 4.5. The above factors point out the importance of combining several techniques during preservation of food in order to prevent unnecessary economic losses due microbial proliferation. The results of this study indicate the potential behavior of model spoilage yeast in water-in-oil emulsions, with respect to liquid oil, solid fat and preservative content. These results should be interpreted with caution when compared with true emulsions as the matrices were simplified in order to separately observe the effect of each forementioned factor.

5.2 FUTURE PERSPECTIVES

Following this study, it is planned to carry out a lipase activity assay for *Candida guilliermondii* to confirm the results obtained from the experiments with solid fat. In addition to this, a complete fatty acid composition analysis and solid fat index of inES 48 hardstock will be carried out. Thereafter, experiments in emulsified and crystallized water-in-oil matrices will be carried out as a representation of various fat table spreads.
LIST OF REFERENCES

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ARROYO, M., ALDRED, D. & MAGAN, N. 2005, Environmental factors and weak organic acid interactions have differential effects on control of growth and ochratoxin A production by Penicillium verrucosum isolates in bread, International Journal of Food Microbiology, 98:223-231


CHENG, H., FRIIS, A. & LETH, T. 2010. Partition of selected food preservatives in fish oil-water
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ANNEX 1

Table 1: Generation times of *Candida guilliermondii* grown at different pH levels as obtained by plate count measurements

<table>
<thead>
<tr>
<th>22°C</th>
<th>YNB</th>
<th>YNB+KS</th>
<th>YNB+OIL</th>
<th>YNB+OIL+KS</th>
<th>YNB+OIL+SOLID FAT*</th>
<th>YNB+OIL+SOLID FAT+KS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>6.0 ± 1.49&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.73 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.70 ± 3.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.41 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.05 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>3.63 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.86 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.27 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.95 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.95 ± 1.30&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>3.47 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.27 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.80 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.94 ± 0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.74 ±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>3.51 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79 ± 0.36&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.97 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.74 ±0.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>7°C</th>
<th>YNB</th>
<th>YNB+KS</th>
<th>YNB+OIL</th>
<th>YNB+OIL+KS</th>
<th>YNB+OIL+SOLID FAT*</th>
<th>YNB+OIL+SOLID FAT+KS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>2.67 ± 0.95&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.21 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.5 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.75±0.02&lt;sup&gt;a&lt;/sup&gt;</td>
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</tr>
<tr>
<td>4.5</td>
<td>0.66 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.10 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.08 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.77±0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.79±0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>0.77 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.05 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.77±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>0.58 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.11±0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.10±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.77±0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.78±0.03&lt;sup&gt;a&lt;/sup&gt;</td>
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</table>

Table 2: Lag times of *Candida guilliermondii* grown at different pH levels as obtained by plate count measurements

<table>
<thead>
<tr>
<th>22°C</th>
<th>YNB</th>
<th>YNB+KS</th>
<th>YNB+OIL</th>
<th>YNB+OIL+KS</th>
<th>YNB+OIL+SOLID FAT*</th>
<th>YNB+OIL+SOLID FAT+KS*</th>
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</thead>
<tbody>
<tr>
<td>3.5</td>
<td>10.36 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>∞</td>
<td>2.74 ± 1.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.46 ± 1.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.95 ± 4.02&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>4.5</td>
<td>2.84 ± 0.73&lt;sup&gt;b&lt;/sup&gt;</td>
<td>∞</td>
<td>3.06 ± 2.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.45 ± 2.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.95 ± 4.02&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
<tr>
<td>5.5</td>
<td>2.92 ± 0.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.13 ± 1.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.65 ± 2.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.47 ± 4.29&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32.95 ± 4.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>3.06 ± 1.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.44 ± 1.60&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.33 ± 1.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.66±1.80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.70 ± 3.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.53 ± 1.87&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means obtained from three replicates with the exception of * and shown ± standard deviation; within a column, means with different superscripts are significantly different at α = 0.05; solid fat experiments differentiated using the interval method. R-square > 90% for all fittings.

<sup>2</sup>∞ denotes an extended lag phase while ■ denotes no growth or no lag phase.
<table>
<thead>
<tr>
<th>7°C</th>
<th>YNB</th>
<th>YNB + KS</th>
<th>YNB + OIL</th>
<th>YNB + OIL + KS</th>
<th>YNB + OIL + SOLID FAT*</th>
<th>YNB + OIL + SOLID FAT + KS*</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>3.78 ± 3.97a</td>
<td>∞</td>
<td>0.69 ± 0.54a</td>
<td>∞</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>3.20 ± 0.51a</td>
<td>∞</td>
<td>1.42 ± 0.49a</td>
<td>10.86 ± 0.53a</td>
<td>2.02 ± 0.67a</td>
<td>∞</td>
</tr>
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<td>2.72 ± 1.03a</td>
<td>6.41 ± 0.09a</td>
<td>0.81 ± 0.24a</td>
<td>2.54 ± 0.15b</td>
<td>1.19 ± 0.65a</td>
<td>2.04 ± 0.43a</td>
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<tr>
<td>6.5</td>
<td>3.72 ± 0.56a</td>
<td>4.28 ± 0.35b</td>
<td></td>
<td>1.11 ± 0.53c</td>
<td>1.00 ± 0.52a</td>
<td>1.38 ± 0.65a</td>
</tr>
</tbody>
</table>

1 Means obtained from three replicates with the exception of * and shown ± standard deviation; within a column, means with different letters are significantly different at \( \alpha = 0.05 \); R-square > 90% for all fittings.

2 \( \infty \) denotes an extended lag phase while ■ denotes no lag phase.

**Figure 1:** An image showing the different matrices used in the experiment (Left: YNB; Center: YNB + sunflower oil; Right: YNB + sunflower oil + inES 48 hardstock)