Efficacy of different disinfectants on isolated and biofilm-associated yeast from a beverage production facility

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Declaration of own work

I, Roshan Aara Abdul, identity number _____ (student number _____), do hereby declare that this research project submitted to the Central University of Technology, Free State, for the Degree Master of Health Sciences in Environmental Health, is my own independent work. This work complies with the code of Academic Integrity, as well as other relevant policies, procedures, rules and regulations of the Central University of Technology, Free State; and has not been submitted before to any institution by myself or any other person for the attainment of a qualification.

…………………………………..

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I certify that the above statement is correct.

…………………………………..

Dr Olga de Smidt (supervisor)

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Abstract

In the food industry, disinfectants are routinely used to sanitize and disinfect product contact surfaces. These disinfectants provide a necessary and required step to ensure that the foods produced and consumed are as free as possible from microorganisms that can cause foodborne illness and spoilage. Disinfection is the reduction, by means of chemical agents and/or physical methods, of the number of microorganisms in the environment to a level that does not compromise food safety or suitability.

Disinfection can be inadequate and cause unwanted issues such as spoilage which can result in product recalls. Factors which can cause inefficient disinfection include: incorrect dilution of chemicals, inadequate contact time, not cleaning the surface before the disinfection stage or not using a suitable disinfectant. Other factors can include and the presence of biofilms which make the process more challenging or the development of resistance to disinfectant actions.

Sometimes, it is thought that microbial resistance is present when actually the organisms are avoiding contact with the disinfectant because a biofilm is present. Biofilms are polysaccharides that allow attachment to most surfaces. Over time, the film becomes enhanced and may contain different species of bacteria or yeast yielding a constant source of contamination which then reflects as ineffective disinfection is taking place.

In a previous study, 20 yeast species were isolated from final concentrated beverage products and on direct food contact surfaces after the disinfection process, alluding to the fact that the disinfection process was insufficient for a processing facility producing concentrated beverages. Eight disinfectants displaying oxidative and non-oxidative modes of action with active ingredients including peracetic acid, didecyl dimethyl ammonium chloride, iodine and potassium iodide, chlorine and oxygen dissolved and volatile secondary metabolites were tested using the minimum inhibitory concentration (MIC) 96 well broth dilution laboratory protocol. The MIC was performed by five minutes contact time on the 20 yeast isolates at uniform growth numbers against the eight disinfectants, followed by incubation and visual determination of the point where

inhibition stopped. The MIC results yielded two disinfectants with didecyl dimethyl ammonium chloride ($DDAC_2$) active ingredient which displayed 100% inhibition at a maximum concentration of 0.1% usage against the 20 yeast isolates tested compared to ranges of 0% to a maximum of only 20% inhibition achieved by the remaining disinfectants.

The two disinfectants, being quaternary ammonium compounds (QAC's) which had only different concentrations of the active ingredient, were then subjected to facility trial protocols for a period of six months to determine if the action displayed in the laboratory results was also applicable on industrial scale application. The facility trial sampling focused on the direct food contact surfaces which would promote biofilm development, namely the filler line nozzles. Sampling included swabs of nozzles, before disinfectant changeover, two weeks, two months and six months after changeover respectively.

Both disinfectants used during the facility trial showed positive results in terms of yeast growth reductions where one disinfectant which contained a higher concentration of $DDAC_2$ in particular showed 94.5% reduction in number of colonies counted obtained from nozzle swabs within two months of use. Both disinfectants would also result in a significant cost saving initiative for the facility.

Culturable yeast diversity changes during the trial were studied using denaturing gel electrophoresis (DGGE). DGGE results provided further information on which yeasts both or one of the disinfectants tested were able to inhibit. These included *Candida sojae*, *Pichia occidentalis, Rhodotorula dairenensis, Sporidiobolus* sp*.* and *Rhynchogastrema noutii* as well as an uncultured isolate. *Lodderomyces elongisporus* and *Kazachstania exigua* however, were not inhibited during the facility trial by both disinfectants.

The industrial trial demonstrated that QAC disinfectant rather than an acid based product yielded better disinfection in the particular facility. The study identified shortcomings in protocols where an unsuitable growth medium was utilized which caused an

underestimation of yeast load present in nozzles. The identification of these aspects will prove advantageous to the facility to improved monitoring protocols and provide a true representation of the yeast load in filler nozzles. In conclusion the more efficient disinfectant with capabilities to better prevent biofilm development was identified. *L. elongisporus* and *K. exigua* were however not inhibited by these disinfectants and further exposure is necessary to determine the extent of tolerance/resistance. The findings of the study will assist to prevent the occurrences of spoilage experienced by the facility resulting in reduced financial losses and brand protection and provides opportunities for further studies.

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List of abbreviations and acronyms

Abbreviation/acronym	Description
μl	Microliter
A	Adenine
ATP	Adenosine triphosphate
a_w	Water activity
BHA	Beta hydroxy acid
BHT	Butylated hydroxytoluene
BLAST	Basic local alignment search
bp	Base pair
BRC	British Retail Consortium
C	Cytosine
CA	Chloramphenicol agar
cfu	Colony forming unit
CIP	Cleaning in place
ClO_2	Chlorine dioxide
CO_2	Carbon dioxide
CODEX	Codex Alimentarius
COP	Cleaning out of place
Cryo	Cryogenic
$DDAC_2$	N,N Didecyl N,N dimethyl ammonium chloride
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid

EFSA	European Food Safety Authority
EO's	Essential oils
et al.	et alia (and others)
FCD	Foodstuffs, cosmetics and disinfectant act
FDA	Food and drug administration
FSA	Food Standards Agency
FSMA	Food Safety Modernization Act
FSMS	Food safety management systems
FSSC	Food Safety System Certification
G	Guanine
GFSI	Global Food Safety Initiative
GRAS	Generally recognized as safe
H_2O_2	Hydrogen peroxide
HACCP	Hazard analysis critical control points
ISO	International organisation for standardization
ITS	Internal transcribed spacer
Kb	Kilobase
lt	Litre
MCS	Master cleaning schedules
MIC	Minimum inhibitory concentrations
ml	Millilitre
NASA	National Aeronautics and Space Administration
nm	Nanometer
NTC	Non-template PCR control

O_2	Oxygen
O_2-	Superoxide anion radical
$°C$	Degrees Celsius
OD	Optical density
$°F$	Degrees Fahrenheit
OH	Hydroxyl
O-PRP	Operational prerequisite programme
PAS	Publicly available specification
PCA	Plate count agar
PCR	Polymerase chain reaction
pH	Logarithmic scale used to specify the acidity or basicity of an aqueous solution
PRP's	Prerequisite programmes
QAC's	Quaternary Ammonium Compounds
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Rotations per minute
rRNA	Ribosomal ribonucleic acid
SABS	South Africa bureau of standards
SANS	South African national standard
SOP's	Standard operating procedures
sp.	Species
SYBR	Nucleic acid stain dye
T	Thymine

TEA	Tris base, acetic acid and EDTA
TMTC	Too many to count
UPGMA	Arithmetic mean algorithm
USDA	United States department of agriculture
UTC	Untreated controls
UV	Ultraviolet
v/v	Volume/volume percent
VF8	General purpose cleaner
w/v	Percent volume
w/w	Percent mass
WHO	World Health Organization
YPD	Yeast extract, peptone dextrose

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Chapter 1

Introduction

1.1 Introduction

Yeasts are unicellular fungi that reproduce asexually mainly by means of budding. They are a heterogenic group of organisms that usually differ from each other in terms of structural and cultural properties. Yeasts can be found in a range of fresh as well as processed foods and industrial raw materials and produce. Yeasts are well known in the food industry for their beneficial role in the fermentation process (beer, bread and wine). However, an unwanted and abundant growth of yeasts in processing can lead to problems in spoilage, quality and safety, which in turn also leads to financial losses (Salo & Wirtanen, 2005).

Spoilage is a consequence of yeast growth in the product. Food components are utilized as growth substrates by the yeasts and are transformed into a vast array of metabolic end products. In this way, the chemical, physical and sensory properties of the food are changed (Fleet, 1992). Yeast spoilage becomes evident to the consumer in many ways, depending on the product (Fleet, 2011). Yeast spoilage is very predictable, principally occurring in those products where bacterial growth is either retarded or prevented by the intrinsic, extrinsic and processing prevail. Without this competition, yeast will grow and spoil the product (Loureiro, 2000). Yeast spoilage has increased in recent years due to lower doses of preservatives and milder preservation processes e.g. the use of little or no chemical preservatives, required for a higher standard of food quality (Snyder & Worobo, 2018).

Spoilage is a severe problem for the food and beverage industry as it renders products unacceptable for human consumption. Due to the large scale at which foods and beverages are produced, the consequence of spoilage will result in severe economic losses (Loureiro & Querol, 2000). The metabolic activity of the spoilage yeast causes irreparable damage to many litres of beverage products every year. Yeast spoilage is a constant threat and a widespread problem in the beverage industry. Spoilage by yeast consists in the visible or detectable alteration of physical and sensorial properties of the

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food or beverage as a result of their activity (Loureiro & Querol, 2000). The most known alterations occur in acid drinks, with or without sugar, and are characterised by abundant gas production, which may deform or blow packages, cloudiness, sediment or pellicle formation, off flavours dominated by a slight fermentation smell (alcohol, carbon dioxide and esters) and off taste (Fleet, 2006).

One of the most important measures to be assessed in order to prevent spoilage in a processing environment is to evaluate the efficacy of the disinfection process, as inefficiencies in the process can eventually lead to spoilage due to spoilage organisms present on direct food contact surfaces after disinfection takes place. Influencing factors include use of the correct disinfectants, making use of appropriate protocols or proper use of other process parameters such as temperature and time (Mazzola *et al.,* 2009). Many factors are of importance when choosing the most suitable and efficient disinfectant. Factors include, the targets of action (e.g. bacteria, mould, fungus, yeast, spores and biofilms), the mode of actions, the pros and cons associated with the use of specific disinfectants such as cost, effects on final products, effectiveness in hard and soft water, corrosive effects, the required possession of certification for suitable use in the food industry, the capabilities of the disinfectant against biofilms as well as safety measures required during use. Efficient disinfection is therefore an imperative factor in averting spoilage in food and beverage products (Loureiro, 2000).

The chosen facility for this study is one which produces millions of litres of concentrated beverages annually. It is a division of a well-known food and beverage conglomerate with processing facilities across the country producing a wide variety of well-known concentrate beverage brands, including variants such as cordials/iced teas, nectars, milk blends and squashes/drinks which are intended for both local and export markets. This facility has experienced reoccurring issues with spoilage in beverages produced caused by yeast, resulting in financial losses and has therefore been chosen to asses measures which can be taken to prevent the occurrence of spoilage due to yeasts.

1.2 Aim

The aim of this study was to investigate the efficacy of different disinfectants on yeasts isolated from the processing environment and determining the effectiveness of selected disinfectants based on yeast enumeration and diversity analysis.

1.3 Objectives

- Evaluation of minimum inhibitory concentrations (MIC) of disinfectants on selected yeasts previously isolated and identified from the facility.
- Testing the most efficient disinfectant/s in a full scale facility setting using routine microbial analysis as well as assessing the effect on possible biofilm associated yeasts on certain equipment.
- Determining if selected disinfectants are suitable for use and providing the facility with options to eliminate or reduce current spoilage issues.

1.4 Chapter layout

Chapter 1: Introduction.

Chapter 2: Literature review.

Chapter 3: Minimum inhibitory concentration testing of selected disinfectants against yeasts isolated from a beverage production facility. Laboratory analysis to determine which of the chosen disinfectants are most effective in inhibiting most or ideally all the chosen yeasts isolates.

Chapter 4: Selected disinfectants beverage facility trial: CIP process and molecular analysis. Full scale facility trial to assess effectiveness of selected disinfectants on biofilm associated with filling equipment. Analysis of yeasts present before, during and after trial.

Chapter 5: Concluding remarks

1.5 References

Fleet, G. (1992). Spoilage Yeasts. *Critical reviews in biotechnology* **12**, 1-44.

Fleet, G.H. (2006). The Commercial and Community Significance. In A. Querol & G. Fleet (Eds.), *Yeasts in Foods and Beverages* (pp. 1–6). Berlin Heidelberg: Springer.

Fleet, G.H. (2011). The yeasts. *Yeast spoilage of foods and beverages.* **5,** 53-63.

Loureiro, L. & Querol, A. (1999). The prevalence and control of spoilage yeasts in foods and beverages. *Trends in food science & technology* **10**, 356- 365.

Loureiro, V. (2000). Spoilage yeasts in food and beverages: Characterization and ecology for improved diagnosis and control. *Food research international* **33**, 247- 256.

Salo, S. & Wirtanen, G. (2005). Disinfectants efficacy on foodborne spoilage yeast strains. *Food and bioproducts processing* **83**, 288-296.

Snider, R.B. & Worobo, R.W. (2018). Fungal spoilage in food processing. *Journal of food protection 81,* 1035-1040.

Mazzola, P.G., Jozala, A.F., Novaes, L.C., Moriel, P. & Vessoni- Penna, T.C. (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and /or sterilizing agents. *Brazilian journal of pharmaceutical sciences* **45**, 241-248.

Chapter 2

Literature review

2.1 Introduction

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The first yeast originated from ancient Egypt hundreds of millions of years ago, and 1500 species are currently identified. Yeasts can be considered man's oldest industrial microorganism. It's likely that man used yeast before the development of a written language. They are estimated to constitute 1% of all described fungal species. Yeasts are chemoorganotrophs, as they use organic compounds as a source of energy and do not require sunlight to grow (Hoffmann *et al*., 2015). Carbon is obtained mostly from hexose sugars, such as glucose and fructose, or disaccharides such as sucrose and maltose. Some species can metabolize pentose sugars such as ribose, alcohols, and organic acids. Yeast species either require oxygen for aerobic cellular respiration (obligate aerobes) or are anaerobic, but also have aerobic methods of energy production (facultative anaerobes) (Deak, 1991).

Yeasts are very common in the environment, and are often isolated from sugar-rich materials (Fleet, 2001). Examples include naturally occurring yeasts on the skins of fruits and berries (such as grapes, apples, or peaches), and exudates from plants (such as plant saps or cacti) (Pitt & Hocking, 1997). Yeasts are able to grow in foods with a low pH (5.0 or lower) and in the presence of sugars, organic acids, and other easily metabolized carbon sources (Deak, 1991). During their growth, yeasts metabolize some food components and produce metabolic end products (Salo & Wirtanen, 2005). This causes the physical, chemical, and sensible properties of a food to change, and the food is spoiled (Loureiro & Querol, 1999).

Yeasts can proliferate in many different varieties of food products such as cheese, butter, cream, yogurt, sausages, sugar syrups, honey, berries, and fruit products, vegetable including pickled cabbage and cucumbers, juice and soft drinks, alcoholic beverages and wines, salad dressings, mayonnaise, confectionaries, jams and jellies as well as bread (Pitt & Hocking, 1997; Salo & Wirtanen, 2005). The most important factors

governing the susceptibility of a product to spoilage by yeasts include, water activity, nutrients and acidity (Deak, 1991). The most common spoilage effects by yeasts include off flavours, souring, gas production, discolouration, swelling of containers and textural changes (Loureiro & Querol, 1999; Fleet, 2001).

Some yeasts are found in association with soil and insects (Mattsson *et al*., 1999). The ecological function and biodiversity of yeasts are relatively unknown compared to those of other microorganisms. Yeasts, including *Candida albicans, Rhodotorula rubra, Torulopsis* and *Trichosporon cutaneum*, have been found living in between people's toes as part of their skin flora (Lahlali *et al*., 2004; Keszhely *et al*., 2008). Yeasts are also present in the gut flora of mammals and some insects and even deep-sea environments host an array of yeasts (Herrera & Pozo, 2010; Wang *et al*., 2012). A summary of yeast associated with food products and impacts thereof are listed in Table 2.1

Table 2.1 Yeast isolate characteristics, sources and significance

Yeast isolate	Sources	Significance	References
Filobasidium capsuligenum	Breweries, soil & fruits	Spoilage & Fermentation	Keszhely *et al*., 2008
Filobasidium uniguttulatum	Human nail & bird droppings	Pathogenic	Mattsson *et al*., 1999
Zygoascus hellenicus	Human blood & fruit juices	Human infection & spoilage	Coetzee *et al*., 2004; Hasejima *et al*., 2011; Ejdys *et al*., 2013
Candida intermedia	Human skin, throat, animal faeces, soil, beer & grapes	Pathogenic & spoilage	Coetzee *et al*., 2004; Hasejima *et al*., 2011; Ejdys *et al*., 2013
Candida parapsilosis	Human stool, domestic animals, insects, soil, soft drinks factories	Pathogenic & spoilage	Takashi *et al*., 1993; Lin *et al*., 1995; Segal *et al*., 1996; Levy *et al*., 1998; Trofa *et al*., 2008
Candida sojae	Defatted soy beans	Not associated with pathogenicity or spoilage	Oyamada *et al*., 2008
Candida quercitrusa	Frass of oak tress & flowers, soil, wastewater from tanning industry	Pathogenic	Xiao *et al*., 2014; Krutzman *et al*., 2011; Westblade *et al*., 2015
Candida spandovensis	Beer & tropical fruits	Not associated with pathogenicity or spoilage	Henninger & Windisch, 1976; Krutzman *et al*., 2011
Candida oleophila	Various food products, plant tissue and water	Biocontrol agent for blue mould in apples and pears	Lahlali *et al*., 2004;Wang *et al*., 2012
Saccharomyces cerevisiae	Beer, bread, wine, & fruit juices	Brewers/Baker's yeast & spoilage	Herskowits, 1988; Boulton & Quain, 2001
Lodderomyces elongisporus	Soft drinks and juice factory environments, cocoa, soil, human fingernail, human blood & baby creams	Pathogenic & spoilage	Deak, 1991; Kurtzman *et al*., 2015
Yarrowia lypolytica	Soil, marine environments and waste waters	Biotechnological yeast utilized in food production	Morgunov *et al*., 2013; Amaro & Nocaud, 2015
Zygosaccharomyces bisporus	Soft drinks, vineyard & winery environments	Spoilage	Lund & Baird-Parker, 2003; Beuchat, 1987; Barata *et al*., 2011
Cryptococcus laurentii	Droppings and cloacal samples of feral pigeons, soil & grapes	Bio pesticide for apple rot	Shankar *et al*., 2006; Banejee *et al*., 2013

Yeast isolate	Sources	Significance	References
Cryptococcus saitoi	Phyllophane & stems of plants, faeces & aquatic habitats	Not associated with pathogenicity or spoilage	Renker *et al*., 2003
Rhodotorula dairenensis	Soil, water, milk, fruit juice & air samples	Pathogenic	Gadanho & Sampaoi, 2001; Nunes *et al*., 2012
Cystobasidium slooffiae	Deep sea environments	Not associated with pathogenicity or spoilage	Minegishi *et al*., 2006
Trichosporon ovoides	Human hair, soil, cabbages, cheese, scarab beetles, parrot droppings & sea water	"White piedra" infection in humans	Haupt *et al*., 1983; Gueho *et al*., 1992
Wickerhamomyces anomalus	Food, beverage & feed product	Biocontrol agent against other fungi	Schneider *et al*., 2012; Sabel *et al*., 2014

2.2 Spoilage

2.2.1 Economic implications of spoilage

It is impossible to make any true estimation of the levels of yeast spoilage, but the cost must run into millions, possibly billions of rands/euros/dollars per year. Whilst any fermentation incidents may be largely reported, visible yeast spoilage is likely to be only partially reported, and spoilage due to off flavours is likely to be grossly underreported. In addition there are probably a multitude of instances of minor growth of yeasts causing slight or no customer-perceptible effects that are never reported (Stratford, 2006). Yeast spoilage has been underestimated until the last century because there are limited foods and beverages where yeasts outcompete spoilage by moulds or bacteria (Hernández *et al*., 2018). All that can be said with certainty is that the true scale of yeast spoilage is in orders of magnitude greater than published data, particularly amongst the less obstructive spoilage yeasts (Hernández *et al*., 2018).

Reports on losses due to spoilage of foods and drinks by yeast are still rare. The incidence of economic cost of outbreaks of yeast spoilage remains unreported for reasons of commercial confidentiality (Fleet, 1992). In documenting the costs of such

outbreaks; consideration needs to be given to many factors such as, the value of the spoiled product, the cost of recall and disposal, successive decreased retailer and consumer purchase of the product due to a tarnished reputation as well as the legal and insurance fees associated with determining responsibility and awarding compensation (Loureiro & Querol, 1999). Usually, numerous parties represented by manufacturers, suppliers of raw and packaging material and retailers are involved in these cases (Loureiro, 2000).

The USDA Economic Research Service estimated that more than ninety-six billion pounds of food in the U.S. were lost by retailers, foodservice and consumers in 1995. Fresh produce and fluid milk each accounted for nearly 20% of this loss while lower percentages were accounted for by grain products (15.2%), caloric sweeteners (12.4%), processed fruits and vegetables (8.6%), meat, poultry and fish (8.5%), and fat and oils (7.1%) (Rawat, 2015).

2.2.2 Intrinsic/extrinsic factors

Microbial contamination can originate from any step along the manufacturing process. Some examples include raw materials, factory environment, packaging and processing equipment. In order to produce microbiologically safe and stable beverages, controlling raw material quality is essential (Guillamó *et al*., 1998). Post-harvest sources for fresh produce include harvesting equipment, rinse water, transport vehicles, processing equipment and human handling. Chemical and physical treatments are usually practiced to ensure the quality of beverage and process water (Burnett & Beuchat, 2001). Water may bring spoilage microbes to the process areas and to the final product if not treated properly. Common sources of yeast in beverages include process waters especially contaminated cooling and rinsing waters (Stratford, 2006). The food safety management systems (FSMS) practiced are therefore of crucial importance where routine microbial monitoring of materials, surroundings, employee hygiene and equipment is required to take place (Lawlor *et al.*, 2009).

Sweeteners and sugar have also been found to be common sources of spoilage by yeasts. Sweeteners used in the beverage industry are typically in the form of syrups. They contain on average 67^oBrix and have a low water activity (a_w) ranging between 0.2 – 0.9 a_w. Mainly, osmophilic yeasts are capable of growing in these syrups. Low water activity (a_w) controls the growth of yeasts, and it is therefore important to prevent condensate formation in syrup storage tanks and/or containers. Drops of condensate water can establish micro-environments with higher water activity (a_w) and can lead to a rapid increase in yeast growth rate (Lawlor et al., 2009).

The facility environment and unhygienic processing equipment such as packaging, filling and capping mechanisms, conveyors, soap, lubrication systems, meters and proportioning pumps and valve seals can also lead to contamination (Donlan, 2002). Although most industries spend thousands of rands/dollars/euros as well as other resources on monitoring and sanitation; sanitary design, cleaning and sanitation procedures favour build–up of spoilage microbes within the facility (Stratford, 2006). This increase the contamination and spoilage risk of final products as microbes have the ability to attach easily onto the manufacturing surfaces (e.g. processing pipes, feeding lines), forming biofilms which are difficult to clean (Sokunrotanak et al., 2012).

2.2.3 Yeasts as spoilage microorganisms in fruit juice

Quality losses in fresh cut fruits and unpasteurized juices can occur as a consequence of microbiological, enzymatic, chemical or physical changes (Boulton & Quain, 2001). Safety and quality losses by microbiological causes are very significant due to two reasons: firstly, because they constitute a hazard to consumers due to the possible presence of microbial toxins or pathogenic microorganisms in the product, and secondly, due to economic losses as a result of microbial spoilage. Many preservation strategies have been traditionally applied to control microbial growth (Raybaudi-Massilia et al., 2009).

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Products which contain high sugar contents are targets for spoilage by yeasts. The characteristics of these products include low pH, low oxygen levels and a high sugar concentration which prevent the growth of most other organisms. However, these characteristics do not inhibit the growth of osmophilic yeasts (Stratford, 2006). Due to the ability to survive in a habitat restricted to a high solute (e.g. sugar) environment, these yeasts are described as being osmophilic or osmotolerant. High sugar foods include jams, honey, sugar syrups, crystallized fruits and fruit juices. The *Zygosaccharomyces* genus are considered the most frequent spoilage yeast in sugary foods and drinks and is responsible for significant economic losses in these industries (Ridawati *et al*., 2010).

The above mentioned produce is prone to spoilage by osmophilic yeasts together with some xerotolerant species due to containing more than 67% sugar (w/w). Osmotolerant yeasts are able to grow in 50% (w/w) sugar and at a water activity (a_w) of 0.88, whereas osmophilic yeasts are capable of growth at 60% sugar (w/w). *Saccharomyces cerevisiae* is considered to be the main spoilage yeast in concentrates, juices and fruit beverages and therefore is considered to be the source of most problems associated with processed fruits (Tilbury, 1980). *Zygosaccharomyces* is a genus associated with the most extreme spoilage yeasts in the food industry. These yeasts are osmotolerant, fructophiles (prefer fructose), which are highly fermentative and extremely preservative-resistant (Pitt & Hocking, 2009). They usually grow at a slow pace, producing off-odours, flavours and carbon dioxide which may cause food containers such as bottles to swell and eventually burst. This yeast has had a long history as spoilage yeasts within the food and beverage industry. This is mainly because these species can grow in the presence of high sucrose, ethanol, acetic acid, sorbic acid, benzoic acid, and sulphur dioxide concentrations, representing some of the commonly used food preservation methods (Brugnoni *et al*., 2007;).

Zygosaccharomyces rouxii is one of the most extreme osmophilic microorganisms known, causing spoilage in sugar syrups and concentrates. *Zygosaccharomyces mellis* is also osmophilic and causes spoilage of honey; furthermore *Zygosaccharomyces bailii*

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and *Z. bisporus* are the main cause for concern in preserved foods due to their phenomenal resistance to preservatives. These yeasts are extremely tolerant to many conditions that are usually detrimental for cell growth, such as high osmotic pressure, high ethanol concentration, low pH values and the presence of weak organic acids and/or various food preservatives (e.g. sulphite, dimethyl dicarbonate), for example, *Z. bailii's* weak organic acid tolerance varies between 375 and 550 mM for acetic acid and between 4.55 and 9.45 mM for sorbic acid, depending on the strain. These concentrations exceed the legally permitted levels for use as preservatives. The osmotolerance of *Z. bailii* is well exemplified by different experiments: for example previous reports showed that the yeast is able to grow in media containing up to 72% glucose (w/v). The marked osmotolerance and the high fermentation capacity worsen the effects of spoilage, since the carbon dioxide (CO_2) generated during alcoholic fermentation has been reported to be responsible for the explosion of canned and bottled foods (Kuanyshev *et al*., 2017).

Some yeasts which have been found to cause spoilage in fruit concentrates include *Candida* spp*., Debaryomyces hansenii, Hansenula* spp*., Rhodotorula* spp*., Pichia* spp., *Dekkera* spp*., Lodderomyces elongisporus, Hanseniaspora* spp*., Issatchenkia orientalis*, *Kloeckera* spp*., Kluyveromyces marxianus, Saccharomyces* spp*., Torulaspora delbrueckii* and *Zygosaccharomyces* spp*.* A wide variety of yeasts have been associated with spoilage of fruit juices and soft drinks, examples are indicated in Table 2.2 (Tilbury, 1980; Steels *et al*., 2000; Pitt & Hocking, 2009).

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Table 2.2: Yeasts frequently isolated from fruit juice and soft drinks (Deak, 2007; Aneja *et al*., 2014; Obasi *et al*., 2014).

Species	Sources
Candida boidinii	Soft drinks
Candida etchellsii	Soft drinks
Candida inconspicua	Soft drinks & Concentrated juices
Candida intermedia	Fruit juices
Candida parapsilosis	Fruit juices
Candida sake	Soft drinks
Candida stellata	Soft drinks & Concentrated juices
Candida tropicalis	Apple juice & soft drinks
Candida lusitaniae	Fruit juices
Debaryomyces hansenii	Fruit juices
Dekkera anomala	Soft drinks
Dekkera bruxellensis	Soft drinks
Hanseniaspora occidentalis	Fruit juices
Hanseniaspora uvarum	Soft drinks & Concentrated juices
Issatchenkia orientalis	Soft drinks, fruit juice & Concentrated juices
Lachancea thermotolerans	Soft drinks, fruit juice & Concentrated juices
Lachancea fermentati	Soft drinks
Lachancea kluyveri	Soft drinks & Concentrated juices
Lodderomyces elongisporus	Soft drinks & Concentrated juices
Pichia anomala	Soft drinks, fruit juice & Concentrated juices
Pichia fermentans	Soft drinks & apple juice
Pichia guilliermondii	Soft drinks & fruit juice
Pichia kluyveri	Fruit juice
Peronospora manshurica	Carbonated orange juice
Rhodotorula glutinis	Fruit juice
Saccharomyces cerevisiae	Soft drinks, fruit juice & Concentrated juices
Zygosaccharomyces bailii	Soft drinks & Concentrated juices
Zygosaccharomyces rouxii	Concentrated juices

Due to the wide variety of possible ways in which yeast can spoil foods and beverages, proper control mechanisms have to be in place (Davenport, 1997). Controls should address all parts of the production process from raw materials to dispatch which the industry normally addresses through the FSMS where the integral aspects of these systems includes hazard analysis critical control points (HACCP) (Sancho *et al*., 2000). One of the most important factors governing proper control or prevention of spoilage

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would be the disinfection process and selection of effective disinfectants (Stratford *et al*., 2000)*.*

2.2.4 Control measures to prevent spoilage

Food preservation is the process of treating and handling food to stop or slow down food spoilage, loss of quality, edibility, or nutritional value and thus allow for longer food storing. Preservation usually involves preventing the growth of bacteria, fungi (such as yeasts), and other microorganisms, as well as retarding the oxidation of fats which cause rancidity (Deak & Beuchat, 1996). A number of methods of prevention can be used that can either totally prevent, delay, or otherwise reduce food spoilage. Preservatives can expand the shelf life of food and can lengthen the time long enough for it to be harvested, processed, sold, and kept in the consumer's home for a reasonable length of time (Taub, 1999). Maintaining or creating nutritional value, texture and flavour is an important aspect of food preservation, although, historically, some methods drastically altered the character of the food being preserved. In many cases these changes have now come to be seen as desirable qualities, as with cheese, yogurt, and pickled onions (Aneja *et al*., 2014).

Freezing is also one of the most commonly used processes for preserving a very wide range of food including prepared foodstuffs which would not have required freezing in their unprepared state (Taub, 1999). Vacuum-packing stores food in a vacuum environment, usually in an air-tight bag or bottle (Aneja *et al*., 2014). The vacuum environment strips bacteria of oxygen needed for survival, thereby slowing spoilage (Deak & Beuchat, 1996).

Sugar is used to preserve fruits, either in syrup with fruit such as apples, pears, peaches, apricots, plums, or in crystallized form where the preserved material is cooked in sugar to the point of crystallization and the resultant product is then stored dry. This method is used for the skins of citrus fruit (candied peel), angelica, and ginger (Deak & Beuchat, 1996). A modification of this process produces glacé fruit such as glacé cherries where

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the fruit is preserved in sugar but is then extracted from the syrup and sold, the preservation being maintained by the sugar content of the fruit and the superficial coating of syrup (Aneja *et al*., 2014). The use of sugar is often combined with alcohol for preservation of luxury products such as fruit in brandy or other spirits (Taub, 1999).

Smoking is used to lengthen the shelf life of perishable food items. This effect is achieved by exposing the food to smoke from burning plant materials such as wood (Taub, 1999). Most commonly subjected to this method of food preservation are meats and fish that have undergone curing (Deak & Beuchat, 1996). Fruits and vegetables like paprika, cheeses, spices, and ingredients for making drinks such as malt and tea leaves are also smoked, but mainly for cooking or flavouring them. It is one of the oldest food preservation methods, which probably arose after the development of cooking with fire (Aneja *et al*., 2014).

Thermal method of heat treatment is another form of preservation. Thermal processes can be classified according to the intensity of the heat treatment. HTLT (temperature 80 °C and holding times >30 s) is the most commonly used method in the processing of juices and beverages; it can be classified as pasteurization (temperature <100 °C), canning (temperature ca. 100 °C), or sterilization (temperature >100 °C. Juice pasteurization is based on a 5 log reduction of the most resistant microorganisms. This method relies on heat generated outside and then transferred into the food through conduction and convection mechanisms (Pertuzzi *et al,* 2017).

Preservative food additives can be antimicrobial. These inhibit the growth of bacteria or fungi, including mould, or antioxidant, such as oxygen absorbers, which inhibit the oxidation of food constituents (Deak & Beuchat, 1996). Common antimicrobial preservatives include calcium propionate, sodium nitrate, sodium nitrite, sulphites (sulphur dioxide, sodium bisulphite, potassium hydrogen sulphite, etc.), and disodium ethylenediaminetetraacetic acid (EDTA) (Taub, 1999). Antioxidants include beta hydroxy acid (BHA) and butylated hydroxytoluene (BHT). Other preservatives include

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formaldehyde (usually in solution), glutaraldehyde (kills insects), ethanol, and methylchloroisothiazolinone (Aneja *et al*., 2014).

All methods of additional ingredients to aid in the preservation of products are however governed by law regarding what is permitted/unpermitted and quantities thereof. Such regulations include those of the Foodstuff, Cosmetics and Disinfectants act 1972, Draft regulations relating to the miscellaneous additives in food (Taub, 1999). Changing methodologies should be done with care to avoid non-compliance in the industry. The use of other preservatives such as sugar and salt also can have a negative influence due to nutritional content perception (Deak & Beuchat, 1996). Consumer awareness is on the increase in this century due to various communication and media sources (Aneja *et al*., 2014).

Consumers relate synthetic preservatives as artificial products resulting in rejection of this type of food processed, so demands for preservatives which have a natural origin have increased drastically (Taub, 1999). The preferable method to assist in the preservation of beverages and food products to prevent microorganisms from being present in the environment and equipment resulting in spoilage is to ensure disinfection is adequately carried out using suitable and legally permitted disinfectants (Deak & Beuchat, 1996).

2.3 Food safety and compliance

2.3.1 Global food safety and compliance

The threat of foodborne illnesses and spoilage has led to the implementation of strict food safety regulations. According to the World Health Organization (WHO) it is estimated that one in 10 people suffer from a foodborne illnesses every year. More worryingly, 420 000 people around the world die each year from such illnesses, including 125 000 children, who are more at risk. With the globalization of food production and supply continuing to increase, manufacturers are under pressure to guarantee their food

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products meet the requirements of not only their own country, but international food safety authorities (Fung *et al.*, 2018). Food safety refers to procedures and regulations to prevent the contamination and poisoning of food products. This necessitates meeting specific requirements in terms of preparation, handling and storage of food, to ensure the risk of foodborne diseases and spoilage is reduced. These factors are enforced by bodies such as the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) (Ralphs, 2018).

In short, the continued growth and success of the global food industry is reliant upon these global testing regulations. Lack of adequate food testing can increase the risk of foodborne illnesses and spoilage, which in turn can lead to the outbreak of disease, resulting in lasting damage to businesses' reputations, as well as costly product recalls or shutdown of production lines (Fung *et al.*, 2018). With the stakes so high, it is no surprise the food safety testing services market is expected to see huge growth over the coming years, forecasted to reach over £12.6 ($17) billion by 2021 (Ralphs, 2018).

Progress in regions where food safety testing is established is expected to anchor this growth, as companies continue to be audited to meet standards under the Global Food Safety Initiative (GFSI), British Retail Consortium (BRC) and the Food Safety System Certification (FSSC) (Fung *et al.*, 2018). The Food Safety Modernization Act (FSMA) in the US in 2011, as well as measures put in place by the Food Standards Agency (FSA) and European Food Safety Authority (EFSA) have increased the demand for food safety testing services in North America and Western Europe. However, developing countries still suffer from a lack of regulation and proper testing mechanisms, which can compromise trade between territories and provide problems for the growth of food safety testing services (Ralphs, 2018).

Taking the FSMA as an example, this act was implemented following several high-profile cases that severely impacted the trust of consumers. The act includes various rulemakings and guidance documents to better guard food products in terms of

prevention tools, inspection and compliance and recall authority. It also founds safety standards that make importers of food products responsible for the quality of imported goods. When it comes to upholding food safety, ignorance is never bliss (Fung *et al*., 2018). All food manufacturers are legally bound to keep all regulations that may be relevant to the foods they produce on file, as well as those related to food safety, should an inspector request them, they must be available and up to date. There are regulations to cover all of the various aspects of food safety, including the recently published regulations governing general hygiene requirements for food premises, the transport of food and related Matters (R.638), published on 22 June 2018 (Hernández *et al*., 2018; Ralphs, 2018).

2.3.2 South African food safety compliance

A lot of focus is based on the international requirements due to global food safety. In South Africa many regulations are also applicable and available to ensure compliance. Food safety and -quality legislation in South Africa is the responsibility of the Departments of Agriculture, Health as well as Trade and Industry. The Department of Agriculture regulates safety and quality of agriculture and animal products in terms of the Agricultural Product Standards act, 1990 (Griffith *et al*., 2017). The Department of Health requires that all foodstuffs shall be safe for human consumption in terms of the Foodstuffs, Cosmetics and Disinfectant act, 1972 (FCD Act). This act addresses the manufacture, labelling, sale and importation of foodstuffs. Matters regarding the hygiene of foodstuffs are addressed by the National Health Act, 2003, and the hygiene requirements at ports and airports including vessels and aircraft are addressed by the International Health Regulations Act, 1974 (Fuller, 2007).

SABS (South Africa Bureau of Standards) is a statutory body that was established in terms of the Standards Act, 1945 (Act No. 24 of 1945) and continues to operate in terms of the latest edition of the Standards Act, 2008 (Act No. 8 of 2008) as the National Standardisation Institution in South Africa, mandated to develop, promote and maintain

South African National Standards (SANS), promote quality in connection with commodities, products and services and render conformity assessment services and assist in matters connected therewith (Griffith *et al.*, 2017). In South Africa the majority of food processing facilities possess amongst others FSSC 22000 certification. FSSC 22000 is an all-inclusive food safety management system standard that incorporates International Organization for Standardization (ISO) 22000, ISO 22003, publicly available specification (PAS 220), Prerequisite programmes (PRP's), HACCP and the application steps of CODEX (The Codex Alimentarius is a collection of internationally recognized standards, codes of practice, guidelines, and other recommendations relating to foods, food production, and food safety). FSSC 22000 certification provides a flexible, risk based global approach to drive continual improvement in food safety and provide confidence across the supply chain (Fuller, 2007).

In order to ensure compliance and produce safe, free of contamination and avoiding spoilage of food products, preventative measures must be implemented. When considering prevention mechanisms, sanitation control is a key concern for food manufacturers (Ralphs, 2018). Key concerns lie in the quality of incoming materials, condition and cleanliness of food-contact surfaces, prevention of cross contamination and the control of employee health and hygiene (Fung *et al.*, 2018). As modern-day processing technologies continue to develop, a range of sanitary and contained food processing equipment is now available to meet food industry regulations. The magnified view on these systems includes a very integral counterpart of FSMS which is HACCP (Hernández *et al.*, 2018).

2.3.3 HACCP within the FSMS

HACCP is a systematic preventive approach to food safety from biological, chemical, and physical hazards in production processes that can render the finished product unsafe and designs measures to reduce these risks to a safe level (Manning & Baines, 2004. In this manner, HACCP attempts to avoid hazards rather than attempting to

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inspect finished products for the effects of those hazards (Hernández *et al*., 2018). The HACCP system can be used at all stages of a food chain, from food production and preparation processes to packaging and distribution (Havelaar, 1994).

HACCP itself was conceived in the 1960s when the US National Aeronautics and Space Administration (NASA) asked Pillsbury to design and manufacture the first foods for space flights. Since then, HACCP has been recognized internationally as a logical tool for adapting traditional inspection methods to a modern, science-based, food safety system (Hernández *et al*., 2018). Based on risk-assessment, A HACCP plans allow both industry and government to allocate their resources efficiently in establishing and auditing safe food production practices (Manning & Baines, 2004). In 1994, the organization international HACCP alliance was established initially to assist the US meat and poultry industries with implementing HACCP, and now its membership has been spread over other professional and industrial areas (Havelaar, 1994).

Within the HACCP system an important control point is effective microbial monitoring to produce good quality food products and prevent spoilage and most importantly food safety outbreaks. In the beverage industry organisms of interest will therefore need to be determined and microbial monitoring should be implemented as part of the HACCP system (Hernández *et al*., 2018). The microbial monitoring in a processing plant will include the environment as well as equipment. Environmental monitoring usually occurs by means of air plates to measure microbial air quality and swabbing to evaluate surfaces such as floors, walls, ceiling, drains, etc (Manning & Baines, 2004). Ingredients and raw materials should be assessed as well as processing aids such as steam, air, gases, chlorine washes and so forth. Final product monitoring also takes place to verify effectiveness of the HACCP system (Havelaar, 1994).

Traditional methods still in use today for the evaluation of equipment microbial quality are surface swabs, either as stick or sponge type. In the beverage industry the organisms of interest would include: general bacteria, yeast and mould, coliforms,

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Escherichia coli, Listeria, *Staphylococcus aureus* and *Salmonella* amongst others (Moretro & Langsrud, 2017). Employee microbial monitoring is also measured by means of hand swabs as they can pose a risk of cross contamination especially where direct food contact takes place during processing. Microbial monitoring is vitally important as we have seen and experienced the most recent food safety listeriosis outbreak in South Africa which resulted in a death toll of over 200 people (de Wet, 2018). Although traditional methods of microbial monitoring are widely used today, advances in technology have also paved the way and many industries have recently amended (Waering & Davenport, 2007).

Rapid cleanliness testing using Adenosine triphosphate (ATP) bioluminescence has become a widely accepted more recent method to monitor the hygienic status of food production lines and verify effective cleaning procedures (Waering & Davenport, 2007). ATP bioluminescence detects microbial cells and food residues, which might persist after inadequate cleaning and provide a source of nutrients for microbial growth although this does come with a price tag as compared to traditional methods (Corbitt *et al*., 2001). When using either swabs or other monitoring tools, samples are taken after the disinfection process to determine and measure effectiveness of disinfection on direct food contact surfaces (Corbitt *et al*., 2001).

2.4 Disinfection and assessment

2.4.1 Disinfection in the food industry

The general methods of disinfection used in the beverage industry are cleaning in place (CIP). CIP is a process allowing a complete system to be cleaned without dismantling it or the manual involvement of the operator. It includes jetting and spraying the surfaces or the circulation of cleaning solutions throughout the equipment with an increased turbulence and flow velocity (Sounrotanak *et al.,* 2012). There are many factors that can influence CIP efficacy, including cleaning chemical concentration, time, cleaning

temperature, cleaning flow rate and the cleaning surface characteristics (Salo & Wirtanen, 2003).

Facilities use COP methods for pieces of equipment and utensils that cannot be cleaned where they are used and must be disassembled, and for pieces of equipment and parts that do not lend themselves to easy cleaning in place (Manning & Baines, 2004). The difference in the two systems can be seen in Figure 2.1. (Tamine, 2009). The primary commercial advantage of CIP processes is a substantial reduction in the time that the plant is unable to produce and the ability to utilise more aggressive cleaning chemicals in a contained environment which cannot be safely handled when compared to manual cleaning (Havelaar, 1994; Hernández et al., 2018).
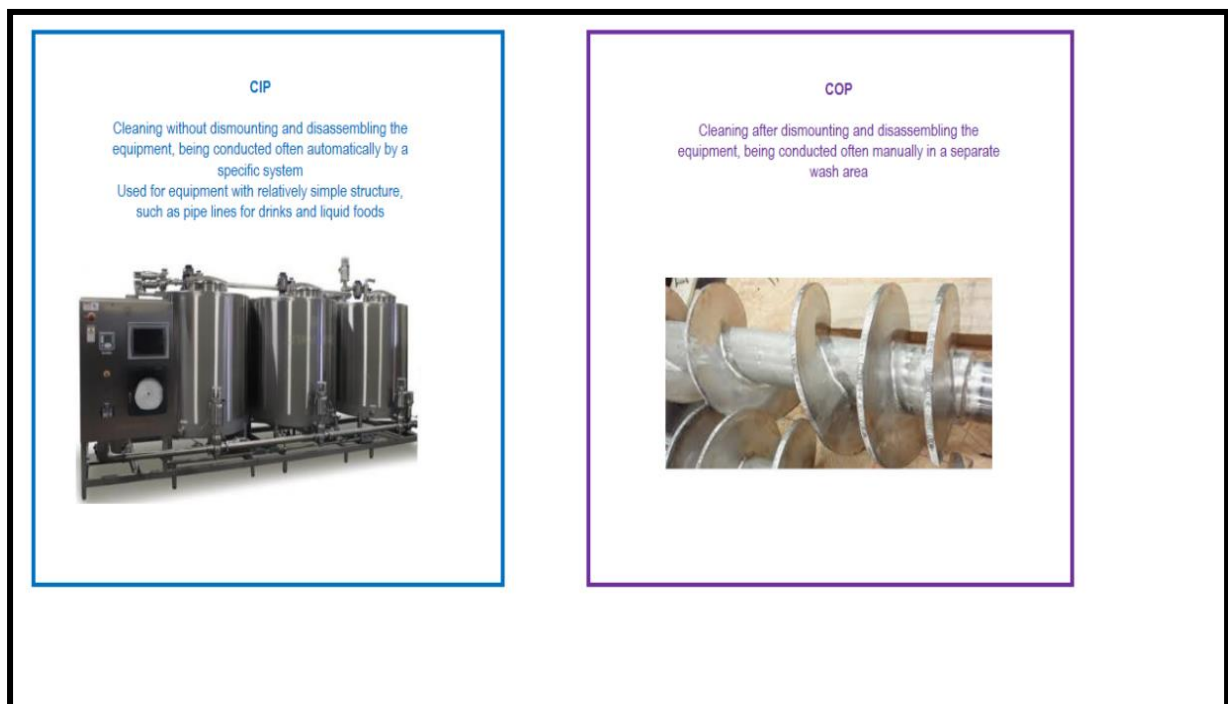
Figure 2.1: Cleaning in place (CIP) vs cleaning out of place (COP)

The design of the tanks should ensure that parts directly above the spray ball are also cleaned and capable of ensuring sufficient circulation (Sounrotanak et al., 2012). Drainage, minimisation of internal probes, crevices, dead ends and stagnant areas, arrangement of valves, couplings and instrument ports and instrumentation should be

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planned carefully so that the equipment is easily cleanable and these features are usually checked and monitored by the ongoing FSMS (Salo & Wirtanen, 2003).

The requirements as set out in the SANS 10049:2012 (Food Safety Management – Requirements for Prerequisite Programmes) standards for equipment design includes the following factors:

1. Facilities with proper drainage shall be provided for the cleaning and disinfection of the premises of the food handling organization and its portable equipment and utensils, and shall be made available at convenient and acceptable points.

2. Equipment for the cleaning and disinfecting facilities shall be constructed of corrosion resistant materials and shall be of a design that is easily cleanable.

3. Such facilities shall be located in a separate room or in a designated area in the preparation, processing and packaging areas where there is an ample supply of cold potable water and hot water, where required, or saturated steam.

 4. Materials used for cleaning and disinfection, hot and cold running water or saturated steam, hose pipes, spray nozzles, brushes, scrapers and any other equipment needed for the cleaning of the food handling organization, and its equipment and utensils shall be made available.

5. These materials and equipment shall not be stored in a room where food handling equipment is stored and shall at no time come into contact with raw materials, the food or their containers or packages.

6. Where used, cleaning in place (CIP) systems shall be designed with sufficient flow rates, contact time and temperature control to allow proper cleaning of the equipment. Where sections of the food contact areas are cleaned during food handling, there shall be a suitable break to atmosphere designed into the piping to prevent the contamination of the food by CIP liquids.

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Attention should also be paid to the quality of the processing water, steam and other additives. Using additives of poor quality easily spoils the process. Furthermore, the tools and methods used must also suit the process and the personnel must be properly trained and responsible to maintain virtuous levels of plant hygiene (Salo & Wirtanen, 2005).

The CIP process includes critical parameters which must be taken into consideration and these include the following:

1. Using the correct vessels for the process, sanitary tank design should include smooth and continuous welds, be self-draining, and consist of internal surfaces that are round or tubular (not flat) to prevent any unwanted accumulation of soil that cannot be removed as shown in Figure 2.2. It's also important for the tanks themselves to be properly vented, self-draining, and have floors capable of fast flushing as shown in Figure 2.3. (Hasting, 2008).

Figure 2.2: An example of a CIP system with all connecting pipelines for dosing, mixing and circulation of disinfectants during disinfection with the required valves, spray balls, hatches and PLC screens for monitoring and control
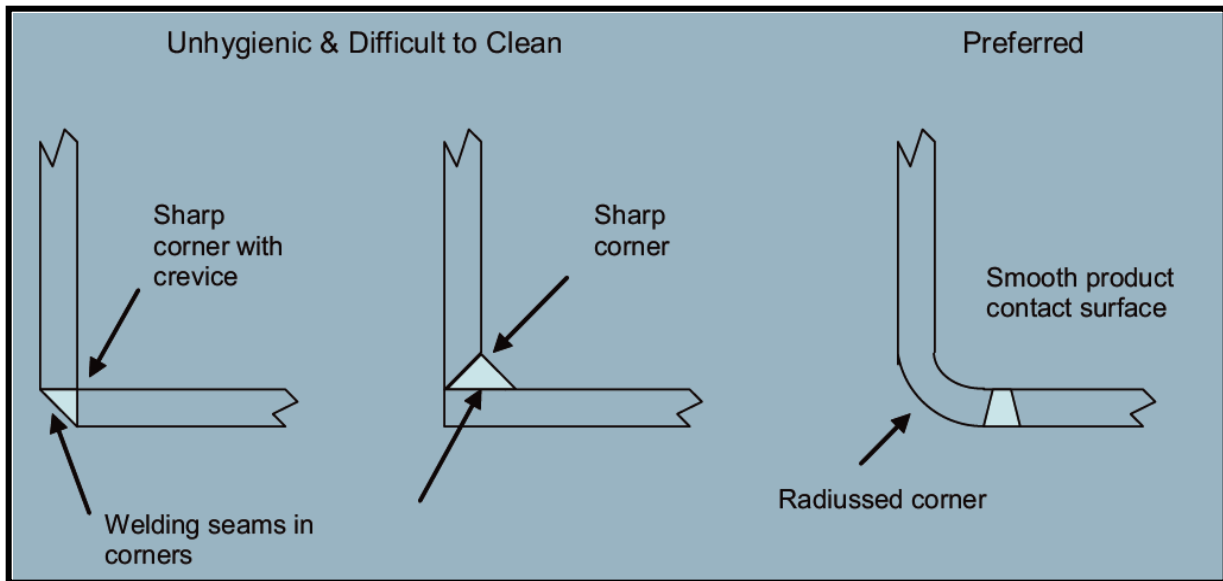
Figure 2.3: Angles and corners of process plant should be well designed to facilitate cleaning (Hasting, 2008).

2. Identify and use the correct disinfectants. Identifying the correct disinfectant for the process is crucial. Some chemicals are highly corrosive to stainless steel and are capable of producing dangerous off-gasses when improperly used, where others are effective against microorganisms when organic matter is present, such as with the processing of poultry or fruit. Some disinfectants are effective in warm and cool water applications or against water hardness films (or milk stone) commonly found in dairy processing (Thomas & Sathian, 2014).

3. Use the correct flow rate. Flow within the system must be at a high enough volume to ensure the flow is turbulent, since turbulence is the mechanical action that "scrubs" interior surfaces of the equipment and piping. To achieve an efficient flow rate, operators should understand their specific processing system. Pump sizes must be sufficient enough for the size of the tank or length of the pipes to be cleaned (Peters, 2015).

4. Monitor and verify. The only way to know if a CIP system is functioning properly is to monitor and validate the system's components. Because CIP systems are usually automated and contain computer-controlled monitoring systems, this is often overlooked (Peters, 2015).

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The selection of detergents and disinfectants in the food industry depends on the efficacy, safety and rinse ability of the agent as well as where it is corrosive or affects the sensory values of the products manufactured (Sounrotanak *et al.,* 2012). An independent quality control system to monitor the cleaning results for a food plant can be integrated in the HACCP program. The key to effective cleaning and disinfection of food plants is the understanding of the type and nature of the soiling agent (sugar, fat, protein, mineral salts, etc.) and the microbial growth to be removed from the surfaces (Salo & Wirtanen, 2005).

Cleaning and disinfection generally occurs in the industry according to the identified requirements of the facility to combat their known problematic organisms, or more often to adhere to the basic principles of ISO, as well as regulatory requirements and chosen certification requirements should be according to customer needs (Vlkova *et al.,* 2008). Equipment requires cleaning and disinfestation at required intervals, and the methodology and results are fully documented. Cleaning programmes also need to be available for facilities, as part of certification, usually known as master cleaning schedules (MCS) (Mazzola *et al.,* 2009).

During monitoring of the effectiveness of disinfectants with the many influencing factors, one other important characteristic which should be obligatory to consider is the ability of the action a disinfectant has against biofilm disruption (Vlkova *et al.,* 2008). Due to the nature of biofilms, the threats associated with having disinfectants which are not effective at targeting biofilms are highly apparent and can lead to having an ineffective disinfection program with disastrous consequences (Mazzola *et al.,* 2009).

2.4.2 Disinfectants and modes of action

There are numerous disinfectants or chemicals which the industry uses to conduct disinfection and cleaning in place, some examples include, sodium hypochlorite, hydrogen peroxide, ozone, paracetic acid, sulphamic acid, persulphate, chlorine releasing agents, alcohols, tensides, foam cleaners, quaternary ammonium compounds (QAC's), chlorhexidine, formaldehyde and ethanol (Salo & Wirtanen, 2005; Sounrotanak *et al.,* 2012).

Disinfectant modes of action are spilt into two different categories where they are either oxidizing or non-oxidizing agents (Table 2.3). Oxidizing disinfectants such as sodium hypochlorite, peracetic acid and hydrogen peroxide attack all cellular material and stop the micro-organism from functioning (Winniczuk & Parish, 1997). Unfortunately they will also attack any food soiling residues thereby reducing the disinfectant efficacy. Non-oxidizing disinfectants such as quaternary ammonium compounds, biguanides and amphoterics are more subtle in their operation, with a different number of modes of action depending on the biocide and organism with certain advantages and disadvantages (Table 2.4) (Penna et al., 2001).

Table 2.3: Disinfectants properties and mode(s) of action of each

Disinfectant	Uses	Modes of action	Active against (as per MSDS/product data sheets)
Peracetic acid	Industrial/institutional sanitizer/disinfectant for previously cleaned Hard Non-Porous food contact surfaces: equipment, pipelines, tanks, vats, filters, evaporators, pasteurizers, and aseptic equipment. Suitable for manual, soak or spray application and for fogging use. Suitable for dairies, wineries, breweries, food and beverage plants, poultry and Egg facilities, and animal housing.	Peracetic acid oxidises and denatures proteins and lipids of microorganisms, leading to disorganisation of the membrane. Swelling may take place in saturation of H + ions, which attract water.	Disperses/penetrates biofilms. Kills bacteria, mould, fungus, and yeast
QAC	General-purpose disinfectant for floors, walls, utensils and other food preparation equipment. Used for open plant cleaning processes and should be applied after surfaces have been thoroughly cleaned and rinsed. Suitable for meat and poultry processors, snack foods, dairies, beverage plants and most other types of food processing operations.	Irreversibly binds to the phospholipids and proteins of the membrane, thereby impairing permeability.	Disinfectant activity against most vegetative forms of micro-organisms including Gram-positive and Gram-negative bacteria and yeasts
Iodine	Widely used as skin disinfectants, particularly before surgery & general-purpose disinfectant for floors, walls, utensils and other food preparation equipment.	Acts by decreasing the oxygen requirements of aerobic microorganisms. Interferes at the level of the respiratory chain of the microorganisms by blocking the transport of electrons through electrophilic reactions with the enzymes of the respiratory chain.	Is rapidly bactericidal, fungicidal, tuberculocidal, virucidal, and sporicidal
Chlorine dioxide	Industrial disinfectant and pesticide for disinfection of water, & equipment in the food and beverage industry.	Acts as an oxidizing agent and reacts with several cellular constituents, including the cell membrane of microbes. By "stealing" electrons from them (oxidation), it breaks their molecular bonds, resulting in the death of the organism by the breakup of the cell.	It has been demonstrated effective as a broad spectrum, anti-inflammatory, bactericidal, fungicidal, and virucidal agent.
QAC	Compound disinfectant currently mostly used in the poultry industry for broad spectrum disinfection of surfaces against poultry viruses, bacteria, fungi, mycoplasma, yeast and algae. It can be used on surfaces as well as air and water. It is effective in the presence of high level organic soiling, at all temperatures, and under hard water conditions.	Irreversibly bind to the phospholipids and proteins of the membrane, thereby impairing permeability, causing cell leakage and death	Active against pathogenic microorganisms and bacteria, poultry viruses, bacteria, fungi, mycoplasma, yeast & algae
Flavouring agents	Flavouring agents but also used as preservative, biocide	Alters cell membrane structure and inhibits respiratory enzymes	Broad antimicrobial properties and can be used to control microbial contamination, against a broad range of organisms such as bacteria, fungi, viruses, protozoa, insects and plants

References (Maris, 1995; Winniczuk & Parish, 1997; McDonald & Russell, 1999; Penna *et al*., 2001; Sola & Wirtanen, 2005; Ioannou *et al*., 2006; Mazzola *et al*., 2009; Trinneta *et al*., 2017)

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Table 2.4: Disinfectants advantages and disadvantages based on required application

Disinfectant	Advantages	Disadvantages
A	Unaffected by hardness and soil. Non-foaming Does not contribute taste, odour or colour Non-corrosive to stainless steel, aluminium Used in cold water disinfection processes	None noted
B	Non-tainting, use on food contact surfaces. Specially formulated for food industry use. Effective in soft or hard water. Non-corrosive. Used in cold water disinfection processes	Foaming agent therefore needing additional water for rinsing after contact time
C	Non-foaming Non-corrosive to stainless steel, aluminium Used in cold water disinfection processes	Moisture Sensitive. Light Sensitive. Air Sensitive. Air causes decomposition of iodine. Affected by hardness and soil Not proven to be effective against spores
D	Non-foaming. Non-corrosive to stainless steel, aluminium Used in cold water disinfection processes The size of a chlorine dioxide gas molecule is 0.124 nm, much smaller than microorganisms and viruses, allowing the gas to easily penetrate into any areas where these microorganisms might be concealed. It is a perfect replacement for chlorine, providing all of chlorine's benefits without any of its weaknesses and detriments such as undesirable pollutants	Will oxidize, ferrous metals and other sensitive materials may be affected Extremely strong odour requiring additional Protective wear during manual handling Before disinfection, cleaning should be carried out using a detergent Short shelf life
E	Cleans and disinfects in one operation and can be used for washing by manual application, dipping /soaking equipment and by spray application. Is effective over a wide pH range and has good performance in the presence of organic matter. It is effective at high and low temperatures and is non-corrosive to equipment. Stays active as long as it is in solution (does not oxidise).	Foaming agent therefore needing additional water for rinsing after contact time Before disinfection, cleaning should be carried out using a detergent
F, G & H	EOs are generally recognized as safe (GRAS) by FDA and, because of their natural origin, are more widely accepted by consumers than "synthetic" alternatives. Non-foaming Non-corrosive to stainless steel, aluminium Used in cold water disinfection processes	Before disinfection, cleaning should be carried out using a detergent Not always water soluble Due to emulsion properties requires more water for rinsing after use and also many forms are not water soluble. Readily oxidized or hydrolysed to give off flavours. Can result in residue and carry-over of taste properties is not rinsed well

References (Maris, 1995; Winniczuk & Parish, 1997; McDonald & Russell, 1999; Penna *et al.*, 2001; Sola & Wirtanen, 2005; Mazzola *et al.*, 2009; Ioannou *et al.*, 2006; Trinneta *et al.*, 2017 & MSDS)

The modes of action have different effects on yeast cells which can result in cell death. Attack of the cell wall is one common mode of action. Cell walls are essential for the survival of yeast cells. Digestion of cell walls in the absence of an osmotic protector leads to cell lysis due to the high internal turgor pressure (Uscanga & Francois, 2003). Thus, substances that interfere with cell wall synthesis may be considered as potential antifungal agents. Because of its rigidity, the cell wall determines the shape of fungal cells. The major components of fungal cell wall are polysaccharides and glycoprotein (Uscanga & Francois, 2003).

The yeast cell wall is made of 30-60% polysaccharides (beta-glucan and mannan sugar polymers), 15-30% proteins, 5-20% lipids and a small amount of chitin. Most of the protein is linked to the mannan-oligo-saccharides and is referred to as the mannoprotein complex. Typically a yeast cell wall contains 15-30% beta-glucan and 15-30% mannan-oligo-saccharides. A basic depiction of the cell wall of a yeast is shown in Figure 2.4. When the cell wall is attacked, lysis occurs causing cell death (Uscanga & Francois, 2003).
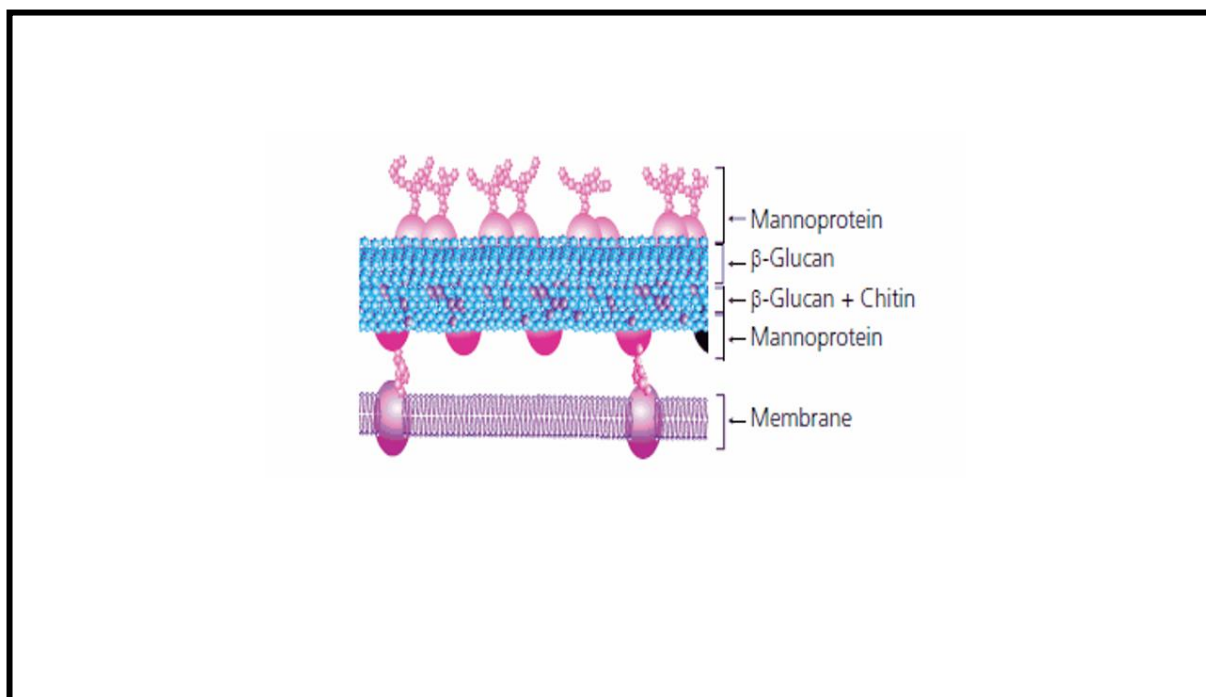
Figure 2.4: Typical cell wall structure of yeast cells (Uscanga & Francois, 2003)

Oxidative stress as a mode of action focuses on the metabolism of yeasts. Yeasts are fungal organisms that can feed on a number of different nutrients, but readily metabolize glucose. They have the ability to metabolize glucose with or without oxygen, and the mechanism of metabolism determines the products formed (Winniczuk & Parish, 1997). Glucose metabolism was chosen to investigate in this study due to it being available in the beverage production environment as sugar in final products. While there are many different species of yeast and they differ metabolically from one another, all can metabolize glucose aerobically. This results in the production of a large quantity of energy, as well as the by-products, carbon dioxide and water (Fiechter & Seghezzi, 1992).

In general, if oxygen is present, yeast will use it to process glucose, since this is much more efficient than metabolizing glucose without oxygen. If there isn't oxygen present, some species of yeast can survive nevertheless, and can metabolize glucose through an alternate pathway that results in the formation of significantly less energy and different by-products. The products of anaerobic metabolism of glucose by yeast are carbon dioxide and ethanol. A basic depiction of metabolism in yeast is shown in Figure 2.5 (Fiechter & Seghezzi, 1992).

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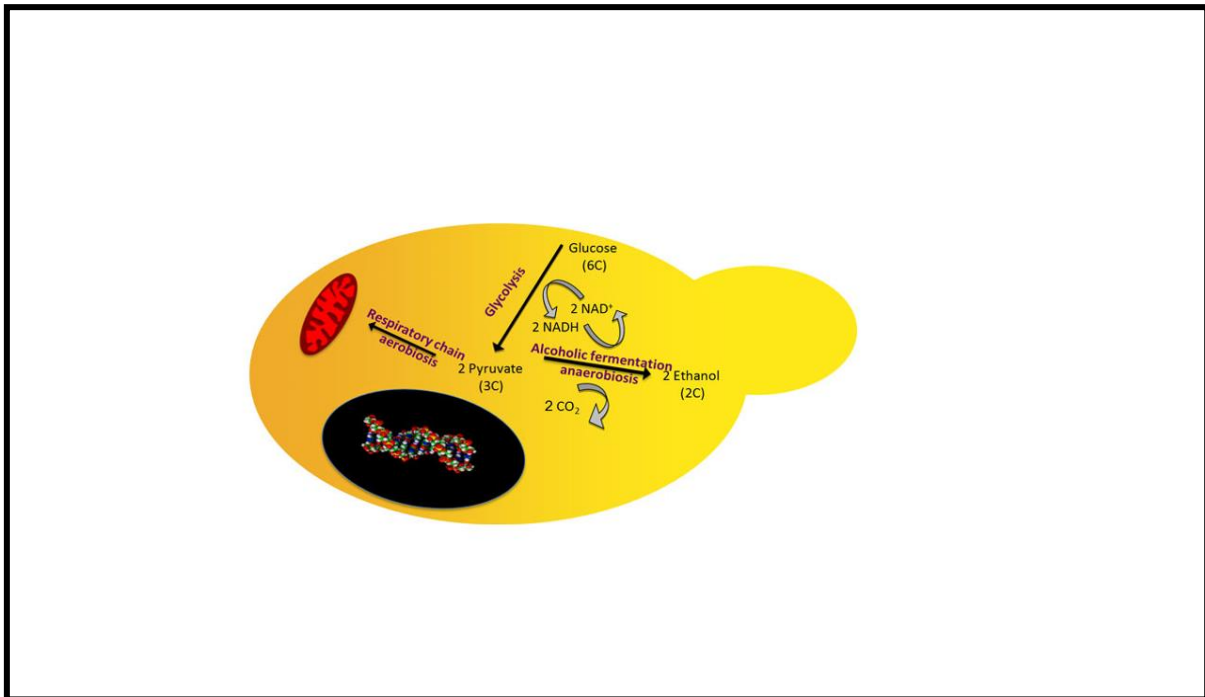
Figure 2.5: Metabolism of yeast, aerobic and anaerobic forms (Fiechter & Seghezzi, 1992)

Reactive oxygen species (ROS) typically arise because of electron leakage from the electron transport chain onto di-oxygen (O_2) during aerobic respiration. Exposure to heavy metals, ultraviolet (UV) irradiation, disinfectants, herbicides, air pollutants, xenobiotics, and other exogenous factors can also induce significant generation of ROS. Failure of cell antioxidant defenses to impede ROS accumulation inevitably results in oxidative stress, a condition broadly defined as an imbalance between pro-oxidants and antioxidants, in favour of the former. This potentially leads to a situation where important cell biomolecules suffer severe oxidative damage, thus compromising the viability of cells. Cell death by oxidation is depicted in Figure 2.6 (Farrugia & Balzan, 2012).
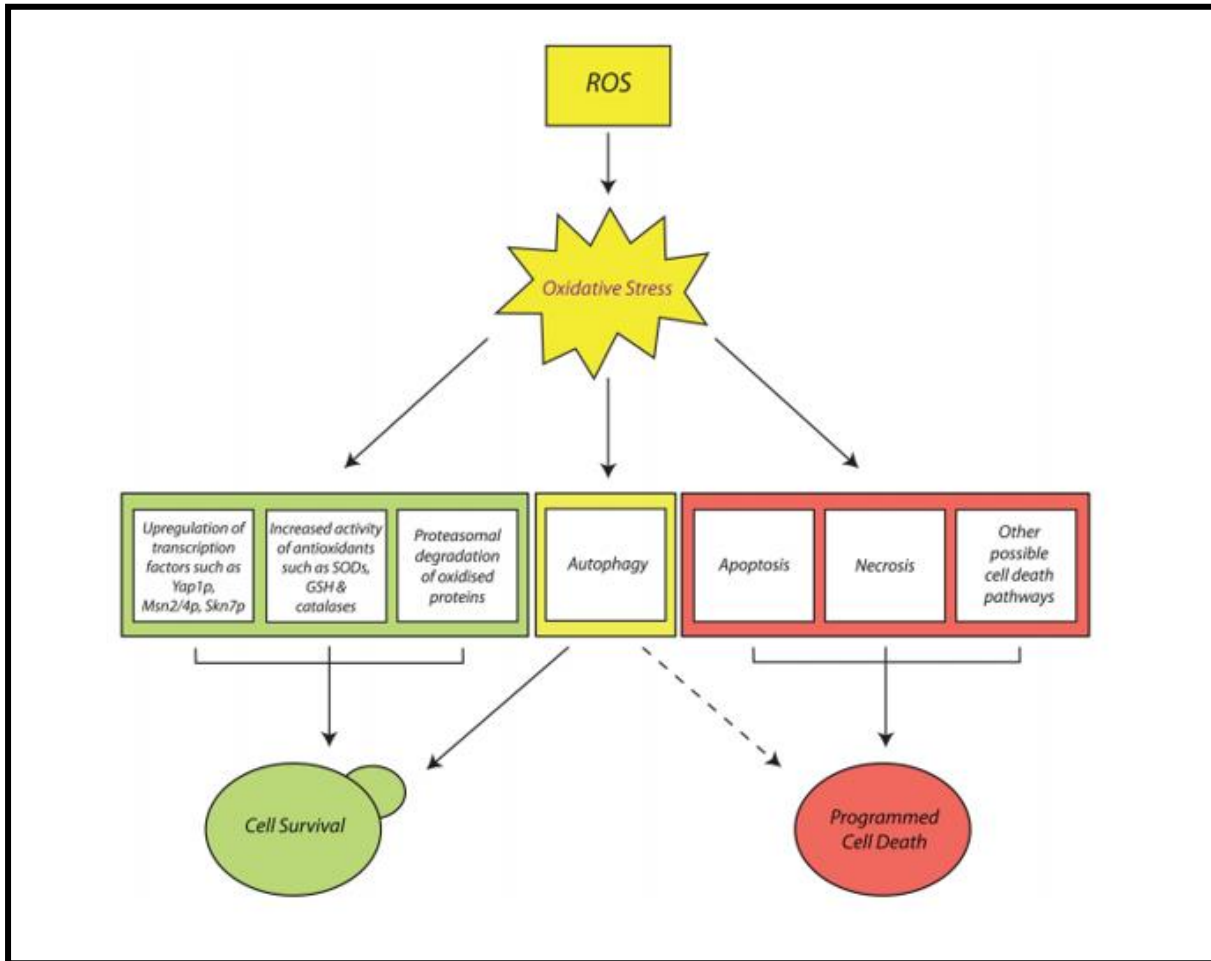
Figure 2.6: Cell death when the yeast cell is under oxidative stress (Farrugia & Balzan, 2012).

QAC's are cationic detergents (surfactants or surface-active agents). They reduce surface tension and form micelles, allowing dispersion in a liquid. The cation portion consists of the central nitrogen with four attached groups, which occur in a variety of structures as seen in Figure 2.7 The negatively charged anion portion is usually chlorine or bromine and is linked to the nitrogen to form the QAC salt (Carmano-Ribeiro & Carrasco, 2013).
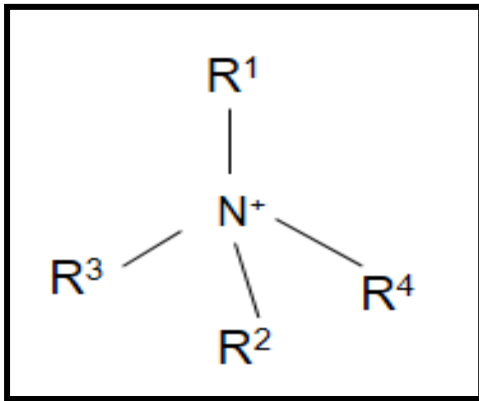
Figure 2.7: Chemical structure of quaternary ammonium compounds (QAC's) (Carmano-Ribeiro & Carrasco, 2013)

QAC's are further classified on the basis of the nature of the R groups, which can include the number of nitrogen atoms, branching of the carbon chain, and the presence of aromatic groups. These variations can affect the antimicrobial activity of the QAC in terms of dose and action against different groups of microorganisms (Carmano-Ribeiro & Carrasco, 2013). The length of the R groups can also greatly affect their antimicrobial activity. Methyl group lengths of C_{12} to C_{16} usually show the greatest antimicrobial activity. Many antimicrobial products contain mixtures of QAC's and other adjuncts to increase their efficacy or to target a specific group of organisms. QAC'S with didecyldimethylammonium chloride with the chemical structure as indicated in Figure 2.8 (Gerba, 2015).
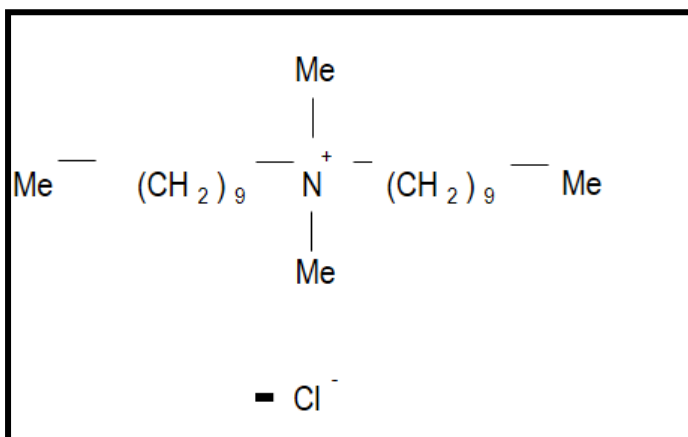
Figure 2.8: Chemical structure of didecyldimethylammonium chloride (Carmano-Ribeiro & Carrasco, 2013)

The modes of actions of disinfectants also influence the ability to attack biofilms. Although the single cell can be destroyed by disinfectants (Mazzola *et al.,* 2009), if disinfectants do not possess the ability to destroy biofilms the problems are never solved. The disinfection process remains inefficient and spoilage yeasts survive and persist due to the protected environment a biofilm creates for individual yeasts cells (Vlkova *et al.,* 2008).

2.4.3 Biofilms in the food industry

Biofilm formation, also referred to as biofouling, is the assemblage of microbial cells attached to the surface and encapsulated in a film or slime layer of extracellular polymers. Biofilms may cause major hygienic complications in the food and beverage industries, because the number of bacteria and yeasts, including spoilage and pathogenic organisms can be very high, and these cells will gradually become detached and cause contamination during production (Gomes *et al*., 2014). Microorganisms in biofilms are closely packed in a matrix that acts as a barrier to cleaning and disinfection, which in turn creates difficulty in removing established biofilms (Sokunrotanak *et al*., 2012). Biofilms can form inside processing equipment and on open surfaces which can lead to spoilage, food safety outbreaks and loss in production efficiency (Jang *et al*., 2017).

Biofilm development is generally characterized as a step wise process. First individual cells attach to the conditioning film in an unstable and reversible manner, where is it still relatively easy to remove the developing biofilm by rinsing. In the next step, the cells secrete polymeric material that binds them more firmly in a heterogeneous, three dimensional structure of extracellular polysaccharides, proteins, nucleic acids, fats and water in which microorganisms are densely packed (Jang *et al*., 2017). This is an irreversible process and now it is difficult to remove the formed biofilm by cleaning. Finally the biofilm begins to disperse cells so they can move on to initiate the formation of new biofilms as can be seen in Figure 2.9. Once biofilm development occurs, removal

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is more challenging and increased pressure is then presented during the cleaning processes (Sokunrotanak *et al*., 2012).
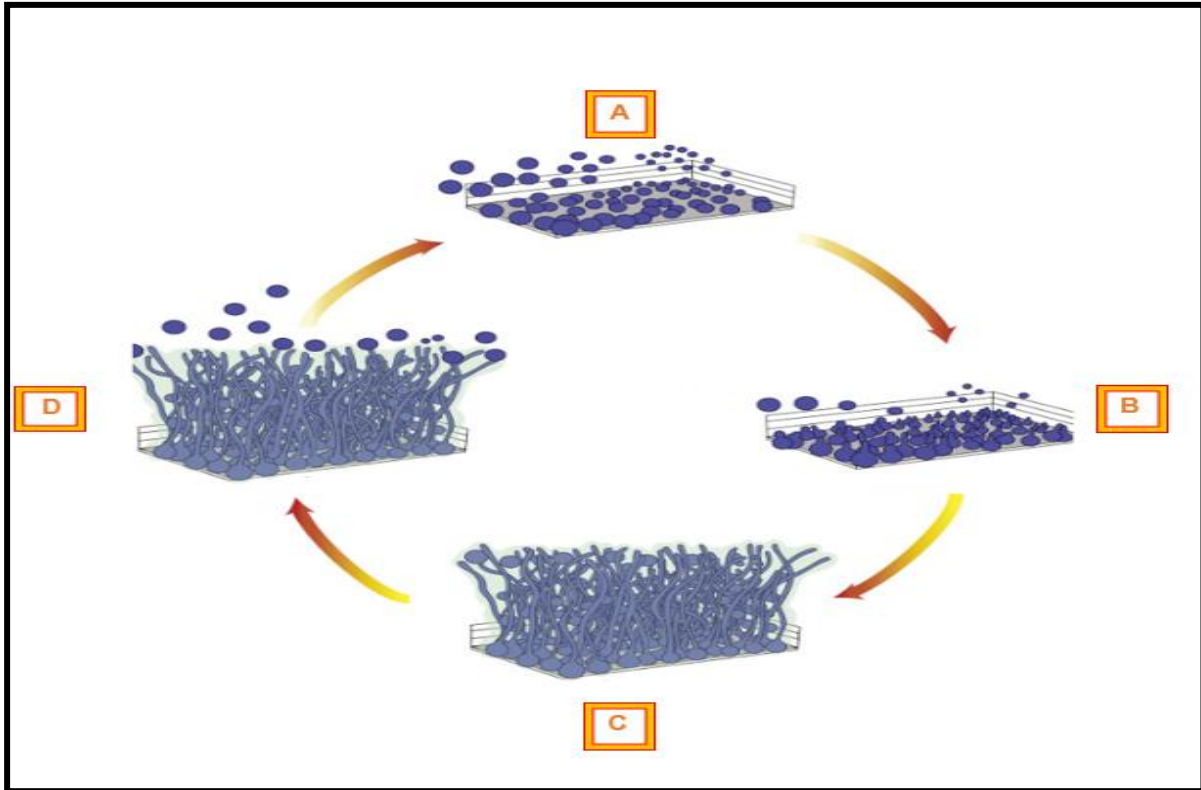
Figure 2.9: Biofilm life cycle. A- Attachment, B- Initiation, C- Maturation and D- Dispersion (Sokunrotanak *et al*., 2012)

Insufficient cleaning can be caused by low cleaning temperature, cleaning flow, cleaning time, or concentration of detergents and disinfectants favouring development of biofilms. Other causes of insufficient cleaning are damaged surfaces caused by corrosion or cracking, and inappropriate hygiene design, for example around manholes, pipe connections and agitators in storage and processing tanks (Sokunrotanak *et al*., 2012). This will often make tanks and containers critical locations in dairies, breweries, wineries and other beverage facilities (Donlan, 2002). Creation of a biofilm can also occur in pipelines, valves and pumps, especially under gaskets and O-rings in joints, nozzle and fittings. The types of equipment prone for biofilm development can be seen in Figure 2.10. In addition, spots with problematic welding are also susceptible to biofilm

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accumulation. Other critical locations include floor drain, doormats, and areas that are difficult to reach in cleaning processes such as under conveyor belts (Jang *et al*., 2017).
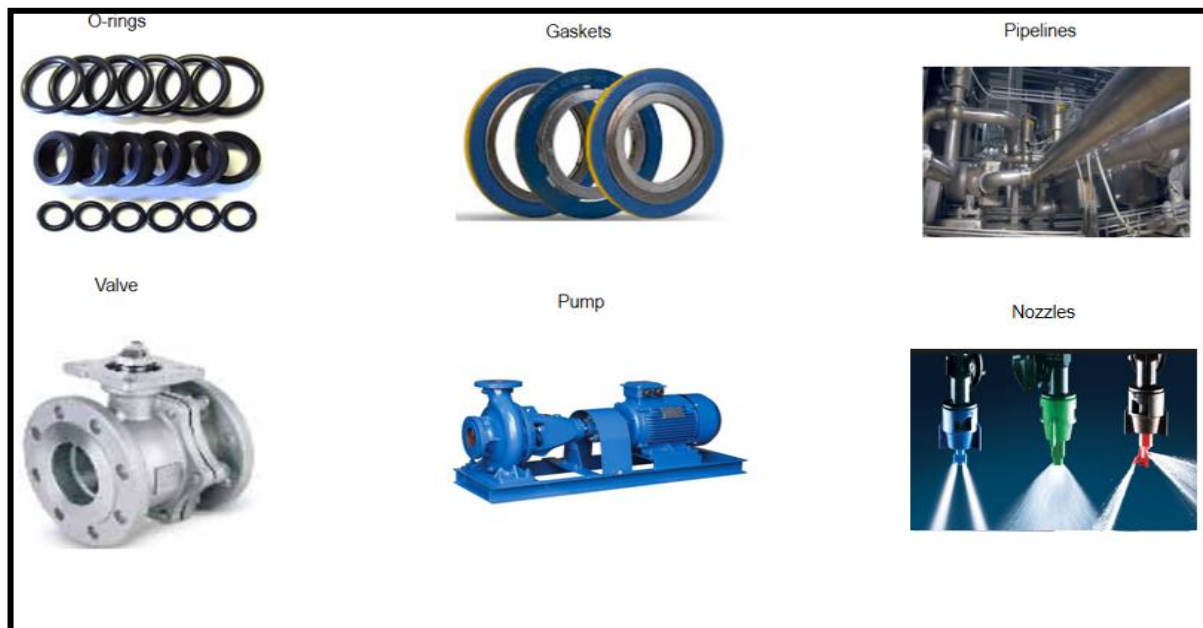
Figure 2.10: Equipment prone to biofilm formation

Yeasts with biofilm formation capabilities include *Rhodotorula rubra, Candida albicans, C. orthopsilosis* and *C. parapsilosis* (Pires *et al.,* 2013). Some bacteria with biofilm formation capabilities include *Bacillus cereus, Escherichia coli, Shigella* spp, *Staphylococcus aureus, Listeria* spp, *Vibrio* spp, *Salmonella epidermis* and *Listeria monocytogenes* (Theraud *et al.,* 2004: Bridier *et al.,* 2015). It is therefore important to also assess areas prone to biofilm formation when evaluating effectiveness of cleaning and disinfection protocols (Vlkova *et al.,* 2008).

2.4.4 Assessment of efficient disinfectants in use

The industry can have cleaning and disinfection procedures in place which are strictly monitored yet still face challenges of spoilage taking place and detecting spoilage microorganisms present in the environment or on equipment (Salo & Wirtanen, 2005). When such instances do occur it is important to assess if the current techniques are justly effective. Taking into consideration the process and materials in use, such as

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disinfectants used. It is also important to change the disinfectants used periodically due to the possible development of resistance/tolerance when one is constantly in use (Winniczuk & Parish, 1997). One of the methods widely used to determine effectiveness of disinfectants used has been identified to be the minimum inhibitory concentration test (Vohra & Poxton, 2011).

Although originally this method was developed to asses antibiotic susceptibility, over the years it has been adapted to also test disinfectants against yeasts and bacteria. The principle of the test is defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after incubation periods (Winniczuk & Parish, 1997). The process can be performed using either test tube or 96 well plate broth dilution methods. The summary of the basic principle of the 96 well plate technique is depicted in Figure 2.11 (Vohra & Poxton, 2011). It is a handy tool which can be used in the industry to determine in vitro whether efficient disinfectants are utilised in the cleaning and sanitation protocols and possibly identify the most effective disinfectant which can be used, especially in cases where a facility has perhaps already identified an ineffective disinfectant currently in use (Salo & Wirtanen, 2005).
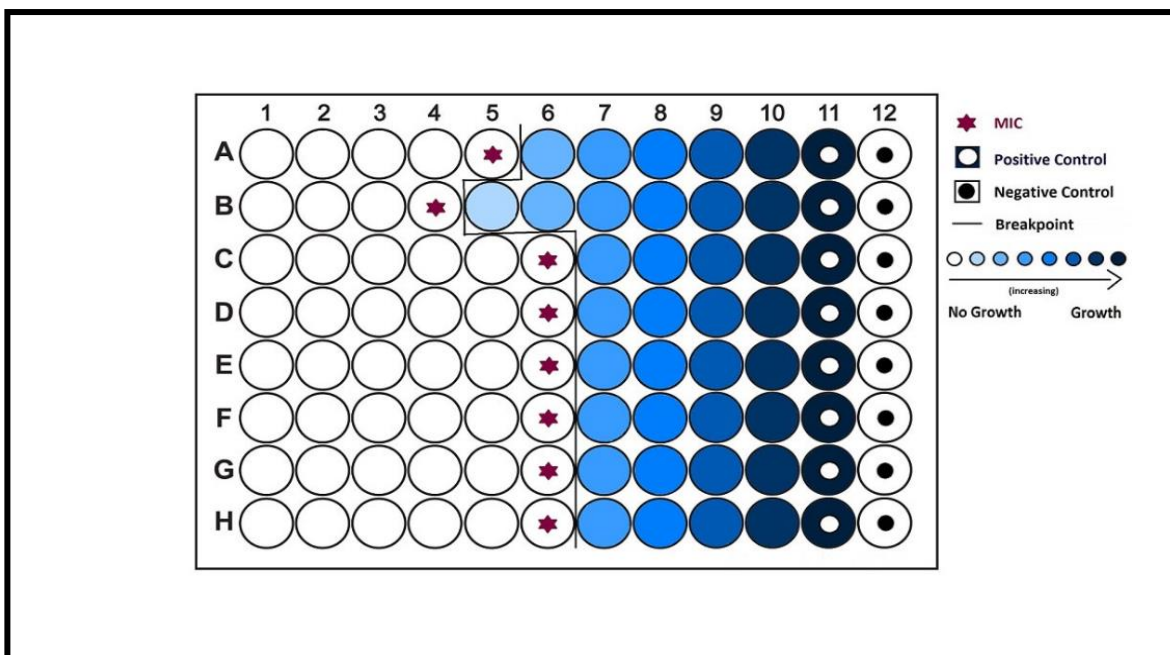
Figure 2.11: 96 well minimum inhibitory concentration testing basic principle (Winniczuk & Parish, 1997).

2.4.5 Large scale determination of efficient disinfection

The methods of in vitro determination such as the MIC 96 well plate method are controlled environments where specific conditions are applied such as a known controlled concentration of a single organism and no contributing environmental factors such as, air, water used, effective and consistent preparation of disinfection dilution if not automated, effects of post process ingredient after rinse before disinfection takes place as well as equipment capabilities of correct functionality (Winniczuk & Parish, 1997). Though the results obtained from these test methods can be promising they can hardly reflect true in house or industrial scale results (Salo & Wirtanen, 2005; Vohra & Poxton, 2011).

An efficient cleaning and disinfection procedure consists of a sequence of rinses using good quality water with application of detergents and disinfectants. Disinfection is required in food plant operations, where wet surfaces provide favourable conditions for the growth of microbes (Salo & Wirtanen, 2003). The efficacy of disinfectants is usually determined in suspensions, which do not mimic the growth conditions on surfaces where the agents are required to inactivate the microbes (Vohra & Poxton, 2011).

The testing of identified efficient disinfectants from in vitro techniques can then be proven when facility testing is performed. This kind of testing will confirm and effectively prove if the in vitro results are reproducible and can provide efficient disinfection in a chosen facility (Hernández *et al.*, 2018). The environment of a specific manufacturing facility can be highly variable and unique as compared to in vivo testing methodology therefore further stressing the importance of conducting said in house testing after in vitro testing is completed (Salo & Wirtanen, 2005).

During and after the in house testing takes place further analysis can then be conducted to paint a final picture in showing the effectiveness of the facility disinfection processes (Salo & Wirtanen, 2005). The results of in house testing such as microbial monitoring plate counts showing log reductions can be facilitated by heat maps etc., and for an in

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depth view of microbial changes can then be seen using molecular applications (Hernández *et al*., 2018).

2.5 Diversity assessment

2.5.1 Molecular analysis

Molecular analysis can be utilized to assess changes in diversity/assortment which perhaps have taken place during the in house testing processes as well as which organisms have been effectively eliminated (Scorjetti *et al*., 2002). Advanced molecular methods are preferred over the traditional approaches, with a few exceptions. Numerous techniques have been developed for species and/or strain identification (Wheals *et al*., 1995). These can be broadly classified into those that require previous growth on culture media (culture-dependent techniques) and techniques that identify yeasts directly from samples (culture-independent techniques) (Hernández *et al*., 2018).

Culture dependent methods are the most extensive molecular techniques for yeast identification (Julien *et al*., 2008). Before the polymerase chain reaction (PCR) amplification step, the yeast isolates are selected by growth on agar media. The most common procedure for selection of the isolates is to choose different colony morphotypes according to their macroscopic examination (colony size, consistency, colour, form, elevation, and margin). So, the diversity of isolates, at least the dominant species, is guaranteed (Labuschagne & Albertyn, 2007). After sampling is complete, the deoxyribonucleic acid (DNA) is extracted and PCR used to amplify and sequence the specific DNA targets (Scorjetti *et al*., 2002).

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2.5.2 Denaturing gradient gel electrophoresis (DGGE) and sequence analysis

DGGE is a technique used for separating DNA fragments according to their mobility's under increasingly denaturing conditions (usually increasing formamide/urea concentrations). Small samples of DNA or ribonucleic acid (RNA) are added to an electrophoresis gel that contains a denaturing agent. The denaturing gel induces melting of the DNA at various stages. As a result of this melting, the DNA spreads through the gel and can be analyzed for single components. DGGE analyses are employed for the separation of double-stranded DNA fragments that are identical in length, but differ in sequence (Muyzer *et al.,* 1993).

In practice, the DNA fragments are usually produced via PCR amplification. The DGGE technique exploits (among other factors) the difference in the stability of G-C pairing (3 hydrogen bonds per pairing) as opposed to A-T pairing (2 hydrogen bonds). A mixture of DNA fragments of different sequences is separated by electrophoresis on an acrylamide gel containing a linearly increasing gradient of DNA denaturants (usually urea and formamide) (Cocolin *et al.,* 2001). In general, DNA fragments richer in GC will be more stable and remain double-stranded until reaching higher denaturant concentrations. Double-stranded DNA fragments migrate better in the acrylamide gel, while denatured DNA molecules slow down or stop in the gel (Hesham *et al.,* 2006). In this manner, DNA fragments of differing sequences can be separated in an acrylamide gel. DGGE is commonly performed for the partial 16S rRNA gene, but also functional genes may be targeted. The gene fragments can be excised from the gel, eluted into sterile water and amplified for sequencing. The relative abundance of various microorganisms can be estimated by measuring the intensity of their bands relative to the intensity of all bands in the corresponding sample used (Muyzer *et al.,* 1993).

Yeast DNA sequencing is widely used in recent years by many researchers to effectively identify organisms and paint a final full picture of yeast being studied and include

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different environments, changes which can take place over time and so forth (Kurtzman & Robnett, 1997). It can effectively be used as a key tool for wholly determining concluding results with ranges in studies being conducted from bacteriophage characteristics, diversity in soil environments and identification of clinically important organisms at much faster speeds than traditional methods of identification (Chen-pei & Padmanabhan, 1973; de Smidt *et al.,* 2014).

The use of DGGE is a very accessible tool due to the fact that identification is provided so the diversity within the testing environment can be established. Although microbial counts are convenient in depicting levels of organisms present, DGGE gives the identity which is vitally important and useful (Olsvik *et al.,* 1992). Knowing the identity of yeasts gives the benefits of determining diversity as well as changes which may take place in an environment over time. Such knowledge can also help to establish if resistant yeasts are present in the biofilm associated equipment to ensure matters are actually addressed and spoilage can be rightly prevented at the end (Pettersson *et al.,* 2009).

Due to accessibility of recent techniques in the industry, analysis is now possible with much shorter time frames and add much value to many studies where identification of multiple strains or organisms are beneficial (Olsvik *et al.,* 1992; Pettersson *et al.,* 2009).

2.6 References

Agricultural Products Standards Act No 119 of 1990. *Regulations relating to the grading of fresh vegetables intended for sales in the republic of South Africa.* Available at https://www.gov.za/sites/default/files/gcis_document/201409/36480rg9961gon364.pdf (Accessed: 8 November 2017).

Amaro, R.L. & Nicaud, J.M. (2015). *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. *Progress in lipid research* **61,** 40–50.

Aneja, K.R., Dhiman, R., Aggarwal, N.K. & Aneja, A. (2014). Emerging preservation techniques for controlling spoilage and pathogenic microorganisms in fruit juices. *International journal of microbiology,* Article ID 758942, 14 pages.

Banerjee, P., Haider, M., Trehan, V., Mishra, B., Thakur, A., Dogra, V. & Loomba, P. (2013). *Crytococcus laurentii* fangemia. *Indian journal of medical microbiology* **31**, 75-77.

Barata, A., Malfeito-Ferreira, M. & Loureiro, V. (2011). The microbial ecology of wine grape berries. *International journal of food microbiology* **153**, 243-259.

Beuchat, L.R. (1987). *Food and Beverage mycology* 2nd edn Springer Dordrecht Heidelberg, London: United Kingdom.

Boulton, C. & Quain, D. (2001). *Brewing yeast and fermentation.* 1st edn John Wiley, London: United Kingdom.

Brugnoni, L.I., Lozano, J.E. & Cubitto, M.A. (2007). Potential of yeast isolated from apple juice to adhere to stainless steel surfaces in the apple juice processing industry. *Food research international* **40,** 332-340.

Burnett, S.L. & Beuchat, L.R. (2001). Human pathogens associated with raw produce and unpasteurized juices, and difficulties in decontamination. *Journal of industrial microbiology and biotechnology* **27**, 104-115.

Carmona-Ribeiro, A.M. & Carrasco, L.D.D.M. (2013). Cationic antimicrobial polymers and their assemblies, *International journal of molecular sciences* **14,** 9906-9946.

Chen-pei, R.W. & Padmanabhan, R. (1973). Nucleotide sequence analysis of DNA XII. The chemical synthesis and sequence analysis of a dodecadeoxynucleotide which binds to the endolysin gene of bacteriophage lambda. *Biochemical and biophysical research communities* **55**, 1092-1099.

Cocolin, L., Aggioa, D., Manzanoa, M., Cantonib, C. & Comi, G. (2001). An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *International dairy journal* **12**, 407-411.

Codex Alimentarius documents. Available at http://www.nicd.ac.za/index.php/codex-alimentarius-documents/ (Accessed: 8 November 2017).

Coetzee, G., Malandra, L., Wolfaardt, G.M. & Viljoen-Bloom, M. (2004). Dynamics of a microbial biofilm in a rotating biological contactor for the treatment of winery effluent. *Water SA* **30**, 406-412.

Corbitt, A.J., Bennion, N. & Forsythe, S.E. (2001). Adenylate kinase amplification of ATP bioluminescence for hygiene monitoring in the food and beverage industry. *Letters in applied microbiology* **30**, 443-447.

Davenport, R.R. (1997). Forensic microbiology II. Case book investigations. *Soft drinks international* **2**, 26–30.

de Smidt, O., Smit, N.J. & Botes, E. (2014). Bacterial diversity in soil from geophagic mining sites in the Qwa-Qwa region of South Africa. *International journal of environmental health research* **25,** 184-195.

De Wet, P. (2018). 'How the deadly strain of listeriosis was traced to Tiger Brand Enterprise polony factory', *Business insider South Africa* 4 March. Available at:

https://www.businessinsider.co.za/Business/how-listeriosis-was-traced-to-tiger-brands-2018-3. (Accessed: 20 December 2018).

Deak, T. & Beuchat, L.R. (1996). Handbook of food spoilage yeast. CRC Press. New York.

Deak, T. (1991). Foodborne yeast. *Advanced applied microbiology 36,* 179-278.

Donlan, R.M. (2002). Biofilms, microbiological life on surfaces. *Emerging infectious diseases* **8**, 881.

Ejdys, E., Biedunkiewicz, A., Dynowska, M. & Sucharzewska, E. (2013). Snow in the city as a spore bank of potentially pathogenic fungi. *Science of the total environment* **42,** 646-650.

Farrugia, G. & Balzan, R. (2012). Oxidative stress and programmed cell death in yeast. *Frontiers in oncology*. **2,** 1-18.

Fiechter, A. & Seghezzi, W. (1992). Regulation of glucose metabolism in growing yeast cells. *Journal of biotechnology*. **27**, 27-45.

Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C. & Maillard, J.Y. (2010). Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms, *Journal of antimicrobial chemotherapy* **65**, 2108–2115.

Fleet, G. (1992). Spoilage yeasts. *Critical reviews in biotechnology* **12**, 1-44

Fleet, G.H. (2001). Spoilage of processed foods: Causes and diagnosis. *Food microbiology group of the Australian institute of food science and technology,* 383–397.

Fleet, G.H. (2006). The Commercial and community significance. In A. Querol & G. Fleet (Eds.), *Yeasts in foods and beverages* (pp. 1–6). Berlin Heidelberg: Springer.

Fleet, G.H. (2011). The yeasts. *Yeast spoilage of foods and beverages*. **5,** 53-63.

47

Foodsftuff, Cosmetics and Disinfectants act 1972. Act No 54 of 1972. *Regulations governing general hygiene requirements for food premises, transport and food related matters.*Available at: http://www.foodfocus.co.za/assets/documents/Regulation%20638.pdf (Accessed: 8 November 2017).

Fuller, L. (2007). Food safety: HACCP awareness and legislation. *South African journal of clinical nutrition* **20**, 2 pages.

Fung, F., Wang, H.S. & Menon.S. (2018). Food safety in the 21st century. *Biomedical journal* **41,** 88-95.

Gadango, M. & Sampaio, P.J. (2001). Polyphasic taxonomy of the basidiomycetous yeast genus Rhodotorula: Rh. glutinis sensu stricto and Rh. dairenensis comb. Nov. *FEMS yeast research* **2,** 47-58*.*

Gomes, L.C., Moreira, J.M.R., Miranda, J.M., Simões, M.,Melo L.F. & Mergulhão, F.J. (2013). Macroscale versus microscale methods for physiological analysis of biofilms formed in 96-well microtiter plates. *Journal of microbiological methods* **95**, 342-349.

Griffith, J., Jackson, L.M. & Lues, R. (2017). The food safety culture in a large South African food service complex: Perspectives on a case study. *British food journal* **119**, 729-743.

Guillamón, J.M., Sabaté, J., Barrio, E., Cano, J. & Querol, A. (1998). Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Archives of microbiology* **169**, 387–392.

Hasejima, N., Kamei, K., Matsubayashi, M., Kawabi, R., Shimura, C., Hijikata, N., Oda, T. & Matsushima, H. (2011). The first case of bloodstream infection by *Candida intermedia* in Japan, the importance of molecular identification. *Journal of infection and chemotherapy* **17,** 555- 558.

Haupt, H.M., Beschorner, W.E., Vaughan, W.P. & Saral, R. (1983). Colonisation and infection of *Tricosporon* species in immunosuppressed host. *Journal of infectious diseases* **2,** 120-126.

Havelaar, A.H. (1994). Application of HACCP to drinking water supply. *Food control*. **5**, 145–152.

Hernándeza, A.F., Pérez-Nevadoa, A.C., S.Ruiz-Moyanoa, M.J., Serradillab, M.C., Villalobosa, A.M. & Córdobaa, M.G. (2018). Spoilage yeasts: What are the sources of contamination of foods and beverages?. *International journal of food microbiology* **286**, 90-110.

Herrera, M.C. & Pozo, M.I. (2010). Nectar yeasts warm the flowers of a winter-blooming plant. *Proceedings of the royal society B: Biological sciences* **277**, 1827–1834.

Herskowitz, I. (1988). Life cycle of budding yeast *Sacchromyces cerevisiae. Microbiology reviews* **52,** 536-539.

Hesham, E.L., Khan, S., XinChun Liu, X., Zhang, Y., Wang, Z. & Yang, M. (2006). Application of PCR–DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil. *Yeast* **23**, 879-887.

Hoffmann, C.F., Wood, V. & Fantes, A.P. (2015). An ancient yeast for young geneticists: A Primer on the *Schizosaccharomyces pombe* Model system. *Genetics* **2**, 403–423.

International Organisation of Standardisation (2015). *ISO 22000:2005 - Food safety management systems -- Requirements for any organization in the food chain*. [online] Iso.org. Available at: http://www.iso.org/iso/catalogue_detail cs number 35466 (Accessed: 8 November 2017).

Jang, H., Rusconi, R. & Stocker, R. (2017). Biofilm disruption by an air bubble reveals heterogeneous age-dependent detachment patterns dictated by initial extracellular matrix distribution. *Npj biofilms and microbioms* **3**, 502-513.

Julien, M.C., Dion, P., Lafreniere, C., Antoun, H. & Drouin, P. (2008). Sources of Clostridia in raw milk on farms. *Applied environmental microbiology* **74,** 6348–6357.

Keszthely, A., Hamari, Z., Pfeiffer, I., Vagvolgyi, C. & Kucsera, J. (2008). Comparison of killer toxin-producing and non-producing strains of *Filobasidium capsuligenum*: Proposal for two varieties. *Microbiological research* **163**, 267-276.

Kuanyshev, N., Giusy M.A., Porro, D. & Branduard. P. (2017). The spoilage yeast *Zygosaccharomyces bailii:* Foe or friend? *Yeast* **34**, 359–370.

Kurtzman, C.P. & Robnett, C.J. (1997). Identification of clinically important ascomycetous yeasts based on nucleotide divergence in the 59 End of the Large-Subunit (26S) ribosomal DNA gene. *Journal of clinical microbiology* **35,** 1216-1223.

Labuschagne, M. & Albertyn, J. (2007). Cloning of an epoxide hydrolase-encoding gene from *Rhodotorula mucilaginosa* and functional expression in *Yarrowia lipolytica*. *Yeast* **24**, 69–78.

Lahlali, R., Serrhini, M.N. & Jijaki, M.H. (2004). Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* against major postharvest diseases of citrus fruits in Mexico. *Communications in agricultural and applied biological sciences* **69**, 601-610.

Lawlor, K., Schuman, J., Simpson, P. & Taormina, J. (2009). In: Sperber, W.H. and Doyle, M.P. (eds.) *Compendium of the microbiological spoilage of foods and beverages*, Food microbiology and safety (pp. 245–2830) Springer: New York.

Levy, I., Rubin, L.G., Vasishtha, S., Tucci, V. & Sood, S.K. (1997). Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. *Clinical infectious diseases* **26,** 1086-1088.

Loureiro, L. & Querol, A. (1999). The prevalence and control of spoilage yeasts in foods and beverages. *Trends in food science & technology* **10**, 356- 365.

Loureiro, V. (2000). Spoilage yeasts in food and beverages: Characterization and ecology for improved diagnosis and control. *Food research international* **33**, 247- 256.

Lund, B. & Baird-Parker, T.C. (2003). Fruit juices, Fruit drinks and Soft drinks. *Microbiological safety and quality of food* 1st edn Springer Dordrecht Heidelberg, London: United Kingdom.

Manning, L. & Baines, R.N. (2004). Effective management of food safety and quality, *British food journal* **106**, 598-606.

Maris, P. (1995). Modes of action of disinfectants. *Revue scientifique et technique (international office of epizootics)* **14,** 47-55.

Mattsson, R., Haemig, P.D. & Olsen, B. (1999). Feral pigeons as carriers of *Cryptococcus laurentii, Cryptococcus uniguttulatus* and *Debaryomyces hansenii. Medical microbiology* **37**, 367-369.

Mazzola, P.G., Jozala, A.F., Novaes, L.C., Moriel, P. & Vessoni- Penna, T.C. (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and /or sterilizing agents. *Brazilian journal of pharmaceutical sciences* **45**, 241-248.

Minegishi, H., Miura, T., Yoshida, Y., Usami, R & Abe. F. (2005). Phylogenic analysis from pectin degrading yeasts from deep sea environments. *Journal of Japanese society of extremophiles* **5,** 21-26.

51

Moretro, T. & Langsrud, S. (2017). Residential bacteria on surfaces in the food industry and their implications for food safety and quality. *Comprehensive reviews in food science and food safety* **16,** 1022-1041.

Morgunov, I.G., Kamzolova, S.V. & Lunina, J.N. (2013). The citric acid production from raw glycerol by *Yarrowia lypolytica* yeast and its regulation. *Applied microbial biotechnology* **97**, 7387-7397.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied environmental microbiology* **59,** 695-700.

National Health Act 2004, No 61 of 2003. *To provide a framework for a structured uniform health system within the Republic, taking into account the obligations imposed by the Constitution and other laws on the national, provincial and local governments with regard to health services; and to provide for matters connected therewith.* Available at: https://www.gov.za/sites/default/files/gcis_document/201409/a61-03.pdf (Accessed: 8 November 2017).

Olsvik, O., Wahlberg, J., Petterson, B., Uhlén, M., Popovic, T., Wachsmuth, I.K. & Fields, P.I. (1992). Use of automated sequencing of polymerase chain reaction generated amplicons to identify three types of Cholera toxin subunit B in *Vibrio cholerae* 01 strains. *Journal of clinical microbiology* **31**, 22-25.

Oyamada, H., Ogawa, Y., Shibata, N., Okawa, Y., Suzuki, S. & Kobayahsi, H. (2008). Structural analysis of cell wall mannan of *Candida sojoe*, a new yeast isolated from defatted soy bean flakes. *Archives of microbiology* **5,** 483-490.

Penna, T.C.V., Mazzola, P.G. & Martins, A.M.S. (2001). The efficacy of chemical agents in cleaning and disinfection programs. *BMC infectious diseases* **16,** 1-16.

Petruzzi, L., Campaniello, D., Speranza, B., Corbo, M.R., Sinigaglia, M. & Bevilacqua, A. (2017). Thermal treatments for fruit and vegetable juices and beverages: A literature overview. *Comprehensive reviews in food science and food safety* **2017**, 688-691.

Pettersson, E., Lundeberg, J. & Ahmadian, A. (2009). Generations of sequencing technologies. *Genomics* **93**, 105-111.

Pires, R.G., Da Silva, J.D.F., Martins, C.H.G., Almeida, A.M.F., Soares, C.P. & Mendes-Giannini, M.J.S. (2013). Effectiveness of disinfectants used in hemodialysis against both *Candida orthopsilosis* and *C. parapsilosis* sensu stricto biofilms. *Antimicrobials agents and chemotherapy* **57**, 2417-2421.

Piterina, A.V. & Pembroke, J.T. (2013). Use of PCR-DGGE based molecular methods to analyse microbial community diversity and stability during thermophilic stages of an ATAD waste water sludge treatment process as an aid to performance monitoring. *Hindawi publishing corporation ISRN biotechnology* **2013**, 1-13.

Pitt, J. I. & Hocking, A.D. (1997). *Fungi and food Spoilage*, 2nd edn Springer Dordrecht Heidelberg, London: United Kingdom.

Pitt, J. I. & Hocking, A.D. (2009). *Fungi and food Spoilage*, 3rd edn. Springer Dordrecht Heidelberg, London: New York.

Prerequisite programmes on food safety for food manufacturing. (2008). *Requirements for prerequisite programmes to assist in controlling food safety risks within the manufacturing processes of international food supply chains.* Available at: https://shop.bsigroup.com/SearchResults/?q=PAs%20220 (Accessed: 8 November 2017).

Ralphs, D. (2018). Ensuring global food safety and hygiene compliance. *New food*, accessed 27 November 2018, <https://www.newfoodmagazine.com/news/73303/food-hygiene-safety>.

Rawat, S. (2015). Food Spoilage: Microorganisms and their prevention. *Asian journal of plant science and research* **5,** 47-56.

Raybaudi-Massilia, R.M., Mosqueda-Melgar, J., Soliva-Fortuny, R. & Martin-Balloso, O. (2010). Control of pathogenic microorganisms in fresh-cut fruits and fruit juices by traditional and alternative natural antimicrobials. *Comprehensive reviews in food science and food safety* **8,** 157-175.

Renker, C., Verena, B., Borstler, B., Heinrichs, J. & Buscot, F. (2004). Diversity of *Cryptococcus* and *Dioszegia* yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores. *FEMS yeast research* **4,** 597-603

Ridawati, B.S.L., Djuwita, I. & Sjamsuridzal, W. (2010). Genetic diversity of osmophilic yeasts isolated from Indonesian foods with high concentration of sugar. *Microbiology Indonesia* **4,** 113-118.

Salo, S. & Wirtanen, G. (2003). Disinfection in food processing – efficacy testing of disinfectants. *Reviews in environmental science and biotechnology* **2**, 293–306.

Salo, S. & Wirtanen, G. (2005). Disinfectants efficacy on foodborne spoilage yeast strains. *Food and bio products processing* **83**, 288-296.

Sancho, G., Gimenez-Jurado, M. & Malfeito-Ferreira, V.L. (2000). Zymological indicators: a new concept applied to the detection of potential spoilage yeast species associated with fruit pulps and concentrates. *Food microbiology* **17**, 613–624.

SANS 10049:2012 Food Safety Management – Requirements for Prerequisite Programmes standards for equipment design. Available at: http://store.sabs.co.za (Accessed: 30 November 2017).

Schneider, J., Rupp, O., Trost, Y., Jaenicke, S., Passoth, V., Goesmann, A., Tauch, A. & Brinkrolf, K. (2012). Genome sequence of *Wickerhamomyces anomalus* DSM 6766 reveals genetic basis of biotechnologically important antimicrobial activities. *FEMS yeast research* **4,** 604-608.

Scorzetti, G., Fell, J.W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS yeast research* **2,** 495–517.

Segal, R., Krtizman, A., Cadivalli, L., Samra, Z., David, M. & Tigva, P. (1996). Treatment of *Candida* nail infection with Terbinafine. *Journal of american academy of dermatology* **35,** 958-961.

Shankar, E.M., Kumarasamy, N., Bella, D., Renuka, S., Kownhar, H., Suniti, S., Rajan, R. & Rao, U.A. (2006). Pneumonia and pleural effusion due to *Cryptococcus laurentii* in a clinically proven case of AIDS. *Canadian respiratory journal* **5,** 275-278.

Sokunrotank, S., Jahid, I.K. & Sang-Do, H. (2012). Biofilm formation in food industries: A food safety concern. *Food Control* **31**, 572-585.

Standards Act, 2008. No 8 of 2008. *To provide for the development, promotion and maintenance of standardisation and quality in connection with commodities and the rendering of related conformity assessment services; and for that purpose to provide for the continued existence of the SABS, as the peak national institution; to provide for the establishment of the Board of the SABS; to provide for the repeal of the Standards Act, 1993; to provide for transitional provisions; and to provide for matters connected therewith.* Available at: https://www.thedti.gov.za/business_regulation/acts/standards_act.pdf (Accessed: 8 November 2017).

Stratford, M. (2006). The yeast handbook. Yeasts in food and beverages. In A. Querol & G. H. Fleet (Eds.), *Food and beverage spoilage yeasts* (pp 335-368). Berlin, Heidelberg: Springer-Verlag.

Stratford, M., Hofman, P.D, & Cole. M.J. (2000). Fruit juices, fruit drinks and soft drinks. In B. M. Lund, A. C. Baired Parker & G. W. Gould (Eds.)*, The microbiological safety and quality of food*, (pp 836- 869). Aspen: Gaithersburg, MD.

Taub, I. A. (1998). Food storage stability. Technology and industrial arts. CRC press. New York.

Theraud, M., Bedouin, Y., Guigen, C. & Gangneux, J.P. (2004). Efficacy of antiseptics on clinical and environmental yeast isolates in planktonic and biofilm conditions. *Journal of medical microbiology* **53**, 1013-1018.

Tilbury, R. H. (1980). Xerotolerant yeasts at high sugar concentrations. In G.W. Gould & J.E.L. Corry (Eds.), *Microbial growth and survival in extremes of environment* (pp. 103-128). Society for applied bacteriology technical series No. 15. London: Academic Press.

Trinetta, V., Morgan, M.T., Coupland, J.N. & Yucel, U. (2017). Essential oils against pathogen and spoilage microorganisms of fruit juices: Use of versatile antimicrobial delivery systems. *Journal of food science* **82**, 471-476.

Uscanga, B.A. & Francois, J.M. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Letters in applied microbiology* **37,** 268-274.

Vlkova, H., Babak, V., Seydlova, R., Pavlik, I. & Schlegelova, J. (2008). Bioflims and hygiene in dairy farms and in dairy the dairy industry: sanitation chemical products and their effectiveness on biofilms a review. *Czech journal of food science* **26**, 309-323.

Vohra, P. & Poxton, I.R. (2011). Efficacy of decontaminants and disinfectants against *Clostridium difficile*. *Journal of medical microbiology* **60**, 1218-1224.

Wang, Y., Wei, A. & Li, H. (2012). Using *Candida oleophila* as a biocontriol agent to prevent foodborne *Escherichia coli* O157 EHEC infections. 2nd edn Springer Dordrecht Heidelberg, London: United Kingdom.

Wareing, P. & Davenport, R.R. (2007). Microbiology of soft drinks and fruit juices. *Chemistry and technology of soft drinks and fruit juices* **2**, 279-297.

Wheals, A.E., Rose, A.H. & Harrison, J.S. (1995): The Yeast, Volume 6, **2nd** edition, Yeast Genetics. London: Academic Press.

Winniczuk, P.P. & Parish, M.E. (1997). Minimum inhibitory concentrations of antimicrobials against micro-organisms related to citrus juice. *Food microbiology* **14**, 373–381.

Chapter 3

Minimum inhibitory concentration testing of selected disinfectants against yeasts isolated from a beverage production facility

3.1 Introduction

Yeasts have the ability to assist in the production of a wide range of foods through the metabolic activity of ethanol fermentation. Ethanol fermentation, also called alcoholic fermentation, is a biological process which converts sugars such as glucose, fructose, and sucrose into cellular energy, producing ethanol and carbon dioxide as a byproduct. Because yeasts perform this conversion in the absence of oxygen, alcoholic fermentation is considered an anaerobic process (Deak & Beuchat, 1996). Ethanol fermentation has many uses, the most commonly known products which use the metabolic activity of yeast fermentation to create beneficial and or profitable food products or drinks are bread, beer and wine. However, yeasts also have the ability to cause deterioration and decomposition of food through fermentation (Deak, 2001).

Yeasts have the ability to invade and grow on many different types of foods and beverages such as grains, nuts, fruits and beans and survive the harvesting and storage process. Some examples of yeasts which have been found to cause spoilage of food products are *Debaryomyces hansenii, Saccharomyces cerevisiae, Zygosaccharomyces bailii, Brettanomyces bruxellensis* and *Zygosaccharomyces rouxii* (Sancho *et al*., 2000; Deak, 2001), These yeasts have been isolated from a variety of products such as yogurt, meat, soft drinks, fruit juices, confectionaries, fruit concentrates, ciders, syrups, wine and others (Aneja *et al*., 2014; Sancho *et al*., 2000; Deak, 2001).

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Fruit juices and soft drinks in particular provide a favourable environment and properties for the growth and survival of yeast due to available water, low pH, high sugar and low oxygen and nitrogen contents. The main ingredients of fruit juices include, amongst others, water, fruit concentrates and pulps or purees, sugar and sweeteners, colourants, flavourants, preservatives and in some cases milk powders, as well as processing aids such as antifoaming agents, glycerol, and stabilisers (Deak, 2001). Fruit juice is more susceptible to yeast spoilage than carbonated beverages and drinks not containing fruit juice (e.g. tonic and cola type product) because they contain a higher amount of nitrogenous rich compounds and vitamins (Aneja *et al*., 2014). A product is considered spoiled when undesired yeast survive, ferment and produce unfavourable by-products which render the specific food or beverage product unsuitable for human consumption (Obasi *et al*., 2014).

Preservation techniques are important in the manufacturing of fruit juices as failure to do so can result in spoilage. Various techniques can be used in the preservation, the most common technique used would be thermal processing, however a major drawback of this technique is usually loss of original taste and flavour. Sodium benzoate, sulphur dioxide and/or potassium sorbate are often used as perservatives in fruit juices, but can be considered a disadvantage since consumers may associate products which contain added preservatives as "artificial products" with a perceived aftertaste and may thus reject the food product (Obasi *et al*., 2014). Futhermore, underdosing of preservative could increase the risk of spoilage as some yeast species may be resistent and still have the ability to proliferate. Yeast resistance to preservatives also poses a threat to the stability of fruit juices. All preservation techniques considered, disinfection procedures are of vital importance to prevent product spoilage (Aneja *et al*., 2014).

Disinfection is an extremely important factor to consider during the manufacturing process of fruit juices. Different methods of disinfection and numerous disinfectants are used in the beverage industry (Theraud *et al*., 2001). The most common form of disinfection in the beverage industry is the process of cleaning in place (CIP): where

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equipment is disinfected without any dismantling (Salo & Wirtanen, 2005). Manual cleaning is still not uncommon for certain parts or processes and will always play an important part of the prevention plans in a food processing facility (Penna *et al.*, 2001).

The selection and correct dosage of efficient disinfectants is therefore very important to ensure proper control of yeast which has the ability to cause spoilage. Spoilage can still occur even when disinfectants are used as yeasts have the ability to develop resistance/tolerance to disinfectants (Penna *et al.*, 2001). Numerous disinfectants are available in the market and used in the beverage industry during the process as part of the prevention plans. Some examples of disinfectants include, quaternary ammonium compounds (QAC's), hypochlorites, acids, alcohols, chlorine dioxide, idiophors and many others. If the disinfectants used do not eliminate or inhibit the growth of yeast, it will result in spoilage of the fruit beverages (Theraud *et al.*, 2001). The causes of ineffectiveness of disinfectants can range from incorrect type of chosen disinfectant or incorrect concentration used as well as not following manufacturers recommendations for usage such as contact time. A widely used technique for assesment of disinfectant efficacy is the mininum inhibitory concentration (MIC) testing process (Salo & Wirtanen, 2005).

The MIC test is a procedure based on the minimum required concentration of the disinfectant needed to cause visible inhibition of the yeast (Winniczuk & Parish, 1997). This tool is an effective technique of determining the correct disinfectant and concentration to use in the process for efficient disinfection and, therefore, prevention of spoilage (Salo & Wirtanen, 2005; Vohra & Poxton, 2011).

The participating facility in this study has experienced spoilage issues associated with yeast species. Although a strict cleaning and disinfection regime is followed, yeasts still persist and cause spoilage incidents. In a previous study twenty yeast species were isolated from the production equipment that were in direct contact with the final product after the CIP process had taken place and created the need for the disinfectant efficacy

testing (Corbett, 2017). The aim of this chapter was therefore to assess the efficacy of currently used and various readily available disinfectants against selected yeast species isolated from this specific beverage production facility.

3.2 Materials and Methods

3.2.1 Fruit beverage facility

The facility in which this study was performed produces concentrated beverages also known as dilutables, where the final usage includes addition of water by the consumer. The facility has five production lines running a variety of different well-known brands of concentrated beverages. Some products contain fruit pulp while others do not. On average this facility produces millions of litres of concentrated beverages per year. The equipment which comes into direct contact with finished products are stainless steel, such as blending tanks, pipelines, filling machines and nozzles. The blending tanks have capacities of 20 000 litres, 10 000 litres or 4 000 litres. The products produced include cordials, ice teas, nectars, milk blends and squashes/drinks. Products are recommended to be stored at room temperature and consumed within 14 days after opening. No additional methods of preservation are used, except for the addition of preservatives and the current disinfection processes as a control measure.

3.2.2 Disinfectants

The disinfectants chosen for this study were readily available and are currently used in the food and beverage sector. They were chosen based on active ingredients, cost, and availability. Samples were provided by local and/or current suppliers of disinfectants for the food and beverage industry (Table 3.1). The disinfectants used were all kept in originally supplied form of packaging and away from sunlight or direct light in a cool environment until use. The disinfectants were prepared according to the recommended concentrations from each supplier by diluting with sterile water obtained from the

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production facility. Sterile water from the participating facility was used for dilution to replicate the conditions at which disinfectants would be diluted in the facility. The facility operates an in house self-developed water treatment system which controls chemical attributes (dosage of chemicals and filtration systems) and sterility using a UV light (Stream line 100 000 UV water sterilizer) to obtain water suitable for the needs of the facility and to adhere to legislation and certification body requirements (FSSC 22000 and SANS 241). The initial concentrations used were based on the principle of MIC testing which requires progressively lower concentrations of disinfectants and were 0.8% for disinfectants A, C, D, E, F, G and H and 1.6% for disinfectant B (Winniczuk & Parish, 1997; McDonald & Russell, 1999; Penna *et al*., 2001; Sola & Wirtanen, 2005; Mazzola *et al*., 2009).

The facility participating in the study is an FSSC 22000 certified plant and therefore all disinfectants used in the experiments required compliance with the requirements of the SANS 22000:2005 (Food safety management systems: requirements for any organisation in the food chain). All the disinfectants had the required certification compliance to SANS 1853:2009 (Disinfectants and detergents disinfectants for use in the food industry). The essential oils used as a possible disinfectant, also posseses SANS 22000:2005 certification, as it is also a food ingredient.

Table 3.1: Descriptive information on disinfectants used in the study

Disinfectant	Active ingredient	Recommended usage concentration	Recommended contact time (min)	Cost per litre in use
A(current)	Paracetic acid, Acetic acid & Hydrogen peroxide	0.3–0.5%	5	R 32.12/L
B	Quaternary ammonium compound (QAC's)	0.5-3%	5	R 24.48/L
C	Iodine & Potassium Iodide	0.1-0.3%	15	R 90.32/L
D	Chlorine & oxygen dissolved	0.01-0.05%	1-3	R 9/L
E	N,N Didecyl N,N dimethyl ammonium chloride (DDAC$_2$)	0.02%	30	R 85/L
F, G & H	Volatile secondary metabolites	0.01%	30	R 400-R 1 000/kg

3.2.3 Yeast isolates and culture preparation

In a previous study, twenty different yeast species were isolated and identified throughout the process flow of this facility (Figure 3.1). Different areas in the facility included raw material storage freezer, blending tanks, filler tanks and nozzles, pipelines as well as spoiled fruit juices. Isolates listed in Table 3.2 were provided as pure cultures cryopreserved in 15% glycerol stored at -80ºC (Corbett, 2017).
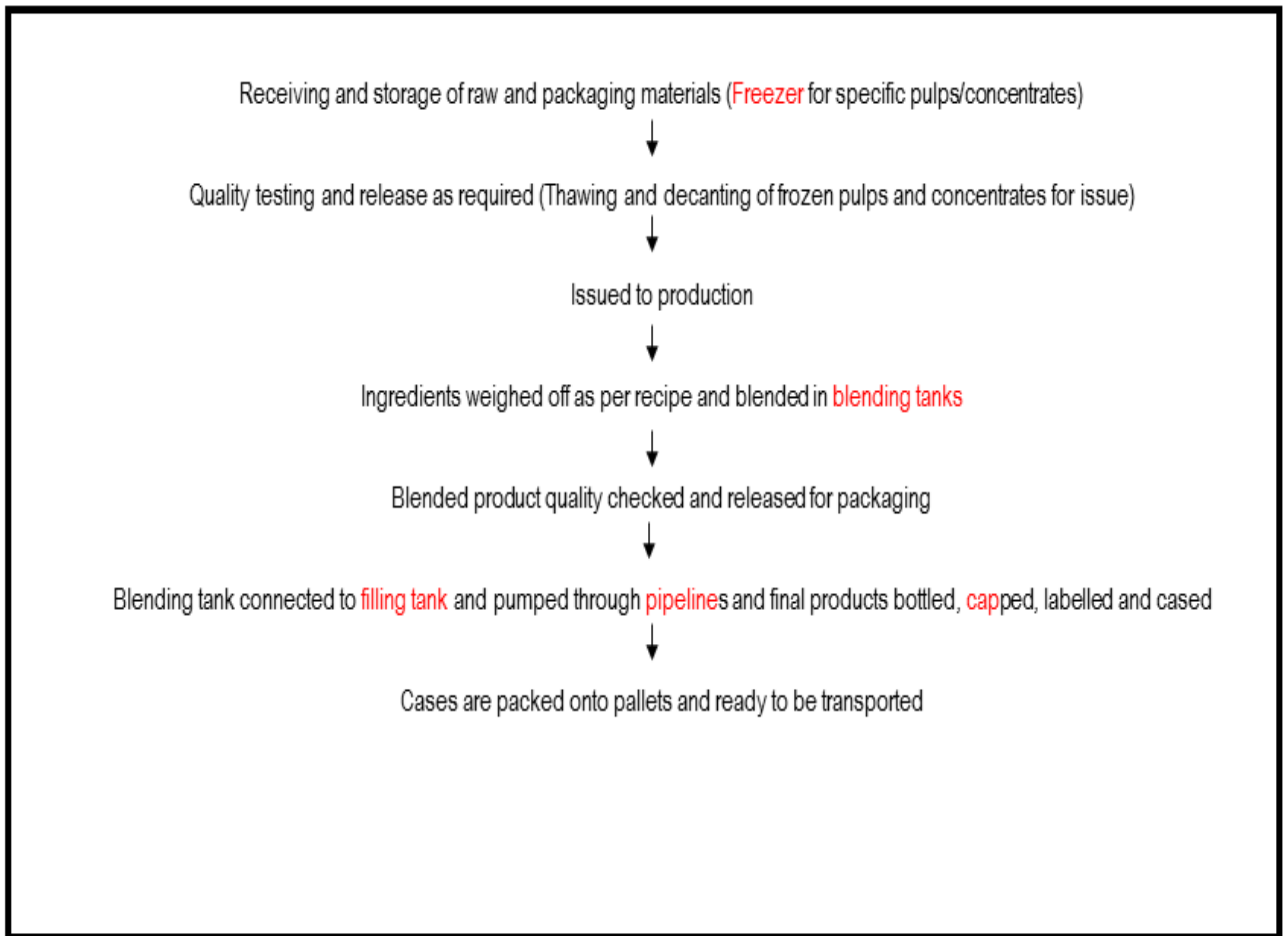
Figure 3.1: Process flow of beverage bottling facility and points of isolation indicated in red.

Table 3.2: Yeast isolates identified by DGGE and points of isolation (Corbett, 2017)

Isolate name	Accession Number	Point of Isolation
Filobasidium uniguttulatum isolate KMC-Y140	KU708234.1	Product filling lines
Candida quercitrusa isolate KMC-Y23	KU708239.1	
Wickerhamomyces anomalus isolate KMC-Y112	KU708244.1	
Naganishia globosa isolate KMC-Y8 [synonym: *Cryptococcus saitoi* (Liu *et al.*, 2015)]	KU708249.1	
Zygoascus hellenicus isolate KMC-Y6	KU708235.1	Final Product
Saccharomyces cerevisiae isolate KMC-Y76	KU708242.1	
Zygosaccharomyces bailii isolate KMC-Y2	KU708246.1	
Zygosaccharomyces bisporus isolate KMC-Y54	KU708247.1	
Candida intermedia isolate KMC-Y35	KU708236.1	Blending tanks & Pipelines
Candida parapsilosis isolate KMC-Y116	KU708237.1	
Candida oleophila isolate KMC-Y88	KU708241.1	
Candida spandovensis isolate KMC-Y57	KU708240.1	
Candida sojae isolate KMC-Y12	KU708238.1	
Lodderomyces elongisporus isolate KMC-Y14	KU708243.1	
Yarrowia lipolytica isolate KMC-Y7	KU708245.1	
Papiliotrema laurentii isolate KMC-Y15 [synonym: *Cryptococcus laurentii* (Liu *et al.*, 2015)]	KU708248.1	
Piskurozyma capsuligena isolate KMC-Y98 [synonym: *Filobasidium capsuligenum* (Liu *et al.*, 2015)]	KU708233.1	
Rhodotorula dairenensis isolate KMC-Y32	KU708250.1	
Cystobasidium slooffiae isolate KMC-Y10	KU708251.1	
Trichosporon ovoides isolate KMC-Y109	KU708252.1	

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Actively growing yeast cultures of 10^6 cfu/ml were prepared as broth solutions (Figure 3.2). Briefly, pure yeast cultures were inoculated onto yeast extract, peptone dextrose (YPD) agar plates from cryo-storage and incubated at 25ºC for 48 hours. Colonies were collected from YPD plates, resuspended in YPD broth and the optical density (OD) was determined at 600_{nm} using the SpectraMaxM2e (Molecular Devices) (Mazzola *et al.*, 2009). The appropriate volume of each culture was added to a 50 ml Erlenmeyer flask containing 20 ml YPD broth to yield an initial OD_{600nm} value of 0.1. Flasks were incubated at 25ºC with shaking for 24-48 hours. After incubation the OD_{600nm} values were again determined, the cultures were diluted in YPD broth to a final OD_{600nm} value of 1 and serially diluted in YPD broth to 10^{-5}. Dilutions were plated on YPD agar plates using the easy Spiral® Pro plater (Interscience) incubated at 25ºC for 48 hours and enumerated using the Scan® 1200 automated colony counter (Interscience) (Vohra & Poxton, 2011).
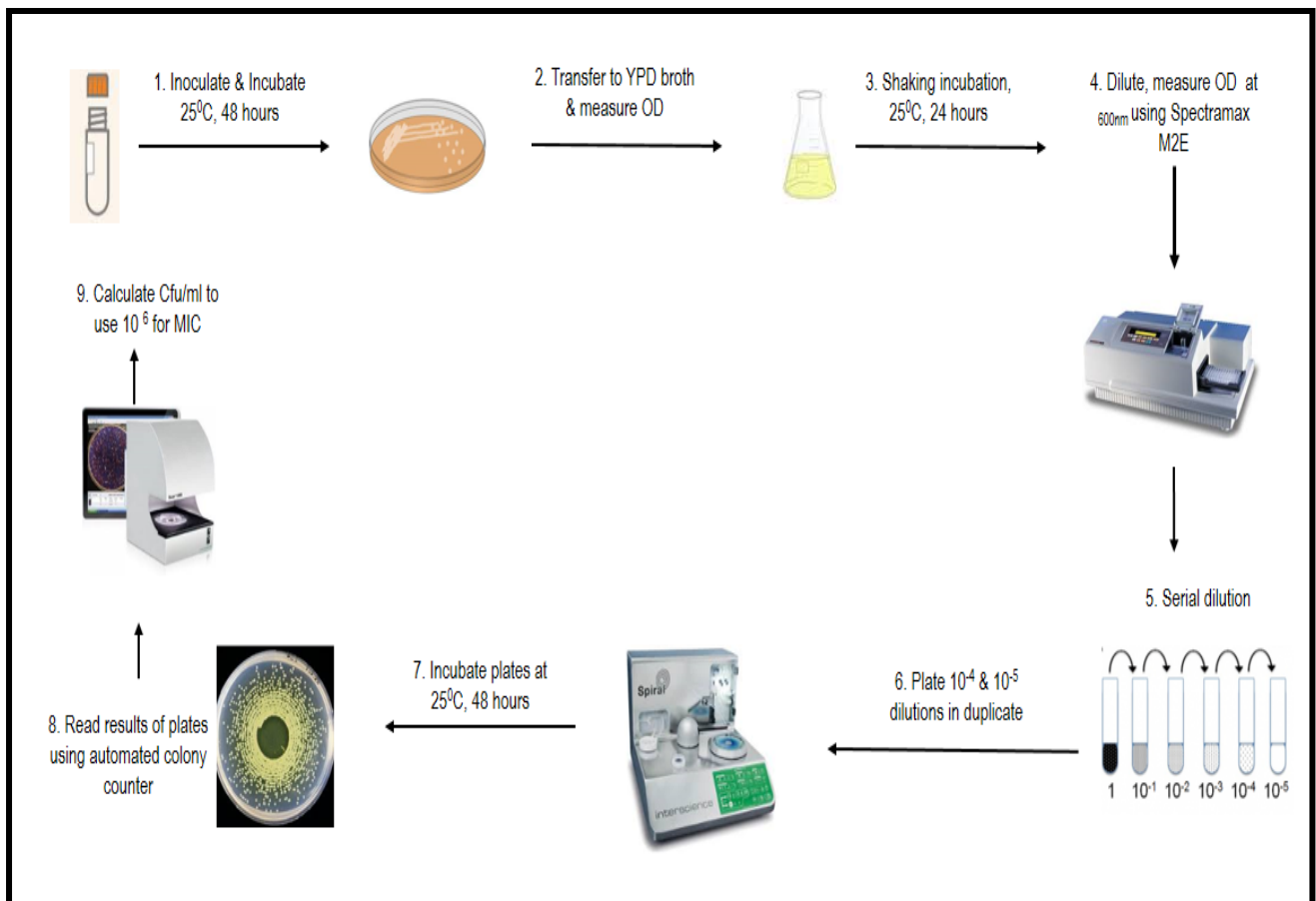
Figure 3.2: Process flow of yeast culture preparation

3.2.4 Minimum inhibitory concentration (MIC) testing procedure

MIC testing can be performed as agar or broth dilution methods. In this study MIC testing was performed in 96 well microtiter plates (Figure 3.3) using the broth dilution method (Rodriquez *et al*., 2002). A 96 well microtiter plate setup was used to accommodate one disinfectant per row at 10 different concentrations against a single yeast isolate. All the yeast isolates listed in Table 2.2 were tested against the disinfectants listed in Table 3.3. Technical replicates were performed in triplicate (Mazzola *et al*., 2009). Two 96 well microplates were used per test; plate 1 (exposure) contained disinfectant and culture while plate 2 (recovery) contained exposed/treated culture in broth. An 8 channel micropipette was used to dispense 100 μl of sterile distilled water into every well in plate 1 and 100 μl sterile broth into every well in plate 2. Then, 100 μl of each disinfectant was added to the first well of every row (A1-H1) in plate 1 (Figure 3.3). Disinfectants were serially diluted 2-fold up to column 10. Broth solution of yeast culture standardized to 10^6 cfu/ml (10 μl) was added to every well from column 1 to column 11 in plate 1. Contact time of 5 min was allowed at 25ºC. Thereafter, 10 μl was transferred from every well in plate 1 to plate 2 using an electronic 8 channel micropipette. Plate 2 was incubated at 25ºC for 48 hours. Plate 1 was discarded. Column 11 was included as untreated controls (UTC) and column 12 as culture medium blanks. After incubation, the growth in plate 2 was assessed; the absence of turbidity (clear broth solution) was considered the MIC for a respective disinfectant and yeast isolate (Vohra & Poxton, 2011).

An important aspect of the MIC test is the required contact time. Disinfectants don't work immediately upon contact with surfaces. Label instructions on disinfectants clearly indicate the amount of time that the disinfectant must remain wet and in contact with surfaces to achieve the desired level to kill pathogens. Some disinfectants take up to ten minutes to kill common pathogens. During this time, surfaces must remain wet. The chosen time used in the test was 5 minutes, as compared to current disinfectant and the

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time which the facility would consider reasonable for disinfection processes to take place (Valeriano *et al*., 2012).
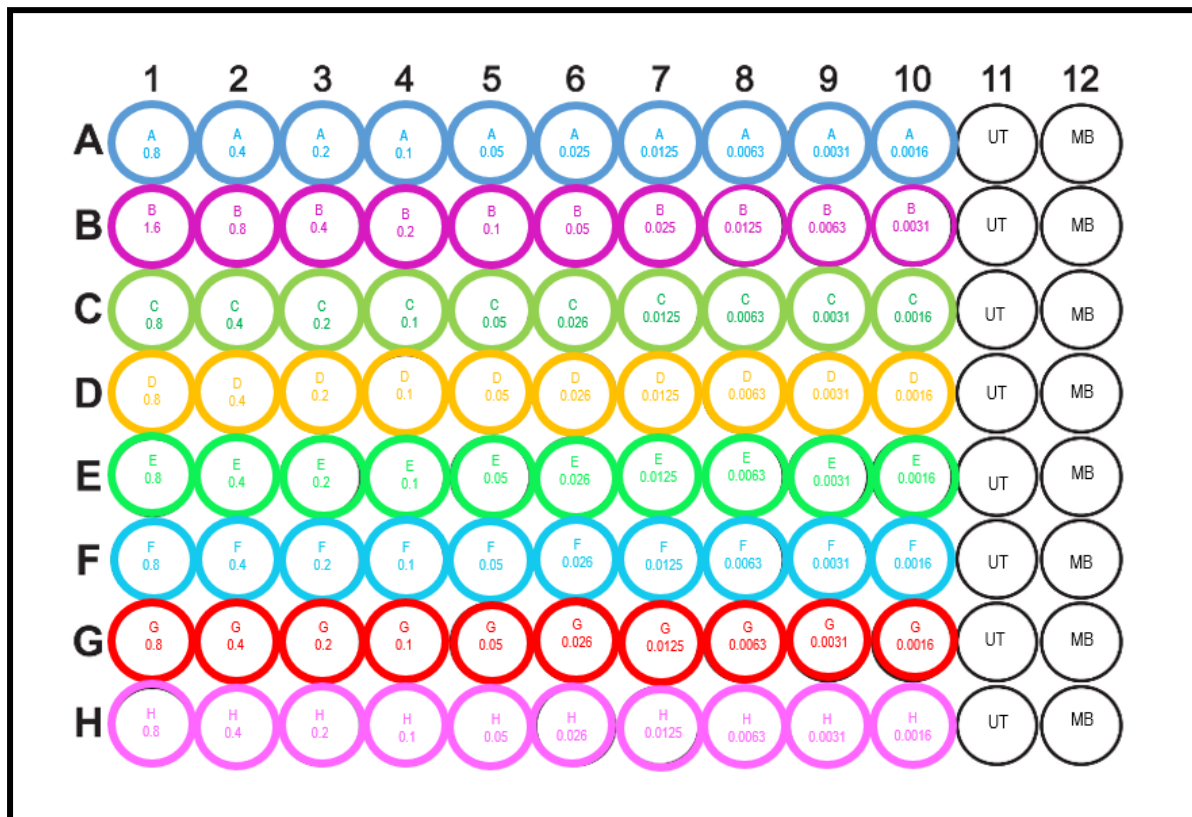
Figure 3.3: Broth dilution method MIC experimental setup. Rows represent different disinfectants and columns (1-10) 2-fold serial dilutions of each disinfectant. Column 11 contained untreated cell suspensions; no disinfectant (UT). Column 12 contained sterile culture medium only (MB).

3.2.5 Isolates characteristics

The results of spoilage by yeast fermentation at the participating facility were a total of 111 logged complaints over a five year period as well as recall of four batches of around 30 000 litres of concentrated beverage containing fruit juice. Within this period research was undertaken to examine the yeast diversity in the facility and a number of different species persistently present in the facility environment and in the spoiled product was isolated and identified (Corbett, 2017) (Table 3.2).

Saccharomyces cerevisiae, Zygosaccharomyces bailii, Zygosaccharomyces bisporus and *Zygoascus hellenicus* are the yeasts which have been isolated from spoiled fruit

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juice from the participating facility and are also yeasts which are generally associated with spoilage in fruit juices. *Filobasidium capsuligenum, Candida intermedia, Candida parapsilosis, Candida quercitrusa, Lodderomyces elongisporus, Yarrowia lypolytica, Cryptococcus laurentii, Rhodotorula dairenensis* and *Trichosporon ovoides* have all been commonly isolated from soil (Haupt *et al*., 1983; Takashi *et al*., 1993; Gadanho & Sampaoi, 2001; Keszhely *et al*., 2008; Xiao *et al*., 2014). *Filobasidium capsuligenum, Candida intermedia, Candida spandovensis, Cryptococcus laurentii, Candida oleophila, Cryptococcus saitoi* and *Candida sojae* have a common source of isolation in fruits and plants (Henninger & Windisch, 1976; Renker *et al*., 2003; Coetzee *et al*., 2004; Lahlali *et al*., 2004; Shankar *et al*., 2006; Keszhely *et al*., 2008; Oyamada *et al*., 2008). *Filobasidium uniguttulatum, Candida intermedia, Cryptococcus laurentii, Cryptococcus saitoi* and *Trichosporon ovoides* have a common source of isolation in droppings or faeces (Gueho *et al*., 1992; Mattsson *et al*., 1999; Renker *et al*., 2003; Hasejima *et al*., 2011; Banejee *et al*., 2013). *Wickerhamomyces anomalus* is generally found in food and beverage products and lastly *Cystobasidium slooffiae* strangely only known to have been found in deep sea environments, was isolated from the facility's in house freezer which operates at a temperature of -22ºC (Minegishi *et al*., 2006; Schneider *et al*., 2012; Sabel *et al*., 2014). The isolate's presence is of no surprise as they have been found in both the final products and facility environment, with common sources such as soil, fruit, droppings and plants. It is comprehensible that these organisms have found their way and managed to inhabit and grow within the facility environment in products.

The facility utilizes pulps, purees and concentrates either as aseptic (pasteurized/ no additional added preservative) or preserved (unpasteurized) as raw materials for processing. Pulps are the membranous content of the fruit's endocarp. The vesicles contain the juice of the fruit. The pulp is usually removed from the juice by filtering it out. Purees retain all of the juice and a large proportion of the fibrous matter naturally found in the fruit. Only excess insoluble fibers are removed while concentrated juice is where the water content from the fruit has been removed. It is the frozen and concentrated form

of fruit juices, which can be kept frozen until use. Taking these points into consideration, the yeasts found in fruit, soil, droppings and plant environments were isolated from the facility. This indicates current measures of disinfection are not effective in controlling yeast contamination in the factory environment and since the sampling was performed after CIP, possible resistance to disinfectant is likely. The yeast isolates are able to adhere, grow, survive and are not effectively inhibited (Corbett, 2017).

The other concerns include those yeasts which have the ability to cause spoilage in fruit juice, as their presence threatens the stability of the products if proper measures of disinfection are not ensured. The other note of concern is *Filobasidium uniguttulatum, Zygoascus hellenicus, Candida intermedia, Candida parapsilosis, Candida quercitrusa, Lodderomyces elongisporus, Rhodotorula dairenensis* and *Trichosporon ovoides* are organisms capable of causing infection and are opportunistic pathogenic to humans, the threat of illness caused due to consumption of products contaminated with these organisms is indeed concerning and undesired, as well as the risks to employees in contact with the product also able to contract illness or infection as well as employees themselves being possible sources of contamination if proper personal hygiene measures are not carried out.

3.2.6 Disinfectants and modes of action

Disinfectants are antimicrobial agents that are applied to the surface of non-living objects to destroy microorganisms that are living on the objects (Maris, 1995). Disinfection does not necessarily kill all microorganisms, especially resistant bacterial spores; it is less effective than sterilization, which is an extreme physical and/or chemical process that kills all types of life (McDonald & Russell, 1999). Disinfectants are different from other antimicrobial agents such as antibiotics, which destroy microorganisms within the body, and antiseptics, which destroy microorganisms on living tissue (Ioannou *et al*., 2006). Disinfectants are also different from biocides; which are intended to destroy all forms of life, not just microorganisms (Sola & Wirtanen, 2005). Disinfectants work by destroying

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the cell wall of microbes or interfering with the metabolism. The benefit of a disinfectant is that it is efficacious at destroying unwanted microorganisms (Penna *et al.*, 2001). A risk of a disinfectant can be its toxicity to humans or its propensity for allowing development of resistance to the active substance (Winniczuk & Parish, 1997).

The modes of action chosen disinfectants used in the study included 1. Disruption of cell membranes (A, B, E, F, G and H), 2. Oxidizing effects on cell metabolism (C & A) and 3. Oxidation (D) (Winniczuk & Parish, 1997). The disinfectants chosen also have advantages and disadvantages which had to be considered for use such as contact time required, if the disinfectants are affected by heavy soil loads, residues which could possibly remain, affectivity at different pH levels, corrosive characteristics and so forth which are summarized in chapter 2 (Sola & Wirtanen, 2005). These parameters are vital to consider when effectively choosing a proper disinfectant, to ensure effective disinfection and no serious implications to the environment and equipment of the facility may arise (McDonald & Russell, 1999).

3.3 Results and Discussion

3.3.1 Growth of isolates

The isolates were grown as described in the methodology and diluted where necessary to obtain 10^6 cfu/ml, which was used for the MIC test. Table 3.7 indicates results of different stages taken in order to obtain uniform growth of isolates to utilize for MIC testing (Winniczuk & Parish, 1997). The methodology used to prepare the cultures for the MIC testing was vitally important as it provided uniformity in growth amounts to conduct a proper MIC test (Salo & Wirtanen, 2005). During the preparation of cultures there was a mixture of some slow and fast growers. Doing the MIC test with cultures without clearly determining the cfu/ml would result in false MIC results especially when working with low concentrations of disinfectants (Vohra & Poxton, 2011).

Amongst the cultures utilized in the study, some were noted to be fast growing, which were reflected in high OD values, as per Table 3.3, some examples include *Filobasidium uniguttulatum, Zygoascus hellenicus, Candida intermedia, Candida quercitrusa, Zygosaccharomyces bisporus, Cryptococcus saitoi* and *Rhodotorula slooffiae* with OD values above 12 after 24 hours. Some examples of slow growers included *Trichosporon ovoides, Lodderomyces elongisporus* and *Wickerhamomyces anomalus* amongst others where the OD values were below 8 after 24 hours of growth in a broth solution. This exercise clearly demonstrated the need for standardization of cultures to obtain uniform growth for use in the MIC testing.

Table 3.3 Isolate growth results during the process of growth rate determination

Isolate	48 hours growth from Cryo (OD_{600nm})	24 hours growth in broth (OD_{600nm})	Average of colonies counted (cfu/ml)
Filobasidium capsuligenum	1.197	11.71	4.40×10^{07}
Filobasidium uniguttulatum	1.972	13.29	6.62×10^{07}
Zygoascus hellenicus	1.332	15.87	8.84×10^{07}
Candida intermedia	0.864	12.25	7.64×10^{07}
Candida parapsilosis	1.422	10.31	7.21×10^{07}
Candida sojae	0.974	11.71	1.05×10^{08}
Candida quercitrusa	0.914	13.57	1.02×10^{08}
Candida spandovensis	0.664	11.55	3.45×10^{08}
Candida oleophila	0.852	11.79	8.35×10^{07}
Saccharomyces cerevisiae	2.124	8.888	5.91×10^{07}
Lodderomyces elongisporus	1.763	6.99	2.65×10^{08}
Wickerhamomyces anomalus	1.346	6.39	3.35×10^{06}
Yarrowia lypolytica	1.295	12.6	2.73×10^{08}
Zygosaccharomyces bailii	1.109	10.89	2.83×10^{07}
Zygosaccharomyces bisporus	0.557	15.36	9.05×10^{07}
Cryptococcus laurentii	1.904	20.43	9.29×10^{07}
Cryptococcus saitoi	1.099	14.37	2.80×10^{07}
Rhodotorula dairenensis	1.763	19.5	9.58×10^{07}
Rhodotorula slooffiae	0.439	12.39	1.08×10^{08}
Trichosporon ovoides	1.137	7.69	5.25×10^{06}

(OD: Optical density. Cryo: Cyropreserved culture, cful/ml: colony forming units per millilitre, nm: Nanometer)

3.3.2 MIC test results

The MIC test results show that disinfectants B and E work extremely well in the inhibition of all 20 isolates as seen in Table 3.4 The remaining disinfecatnts only demonstrated inhibtion of 10%, 15% and 20 % of isolates, or in some cases no inhibition was achieved at the concentrations tested. An example of the 96 well MIC test result plate obtained is shown in Figure 3.4 where inhibition is clearly seen for disinfectants B, C and E and no inhibition for the remaining disinfectants.

Table 3.4 MIC test results showing inhibition concentration of disinfectants against yeast isolates

Yeast Isolates	Disinfectants & Inhibition concentration (%)							
	A	B	C	D	E	F	G	H
Candida intermedia	0.8	0.1	-	-	0.05	-	-	-
Candida spandovensis	0.8	0.1	-	-	0.0125	-	-	-
Zygoascus hellenicus	0.8	0.05	-	-	0.0125	-	-	-
Candida parapsilosis	0.8	0.1	0.8	-	0.025	-	-	0.8
Filobasidium capsuligenum	-	0.2	0.8	-	0.013	-	-	0.4
Wickerhamomyces anomalus	-	0.05	0.8	-	0.05	-	-	-
Candida oleophila	-	0.1	-	-	0.025	-	-	-
Lodderomyces elongisporus	-	0.025	-	-	0.0125	-	-	-
Cryptococcus laurentii	-	0.1	-	-	0.025	-	-	-
Rhodotorula dairenensis	-	0.05	-	-	0.025	-	-	-
Cystobasidium slooffiae	-	0.05	-	-	0.025	-	-	-
Trichosporon ovoides	-	0.1	-	-	0.0125	-	-	-
Yarrowia lipolytica	-	0.1	-	-	0.031	-	-	-
Candida sojae	-	0.1	-	-	0.05	-	-	-
Saccharomyces cerevisiae	-	0.2	-	-	0.05	-	-	-
Zygosaccharomyces bailii	-	0.5	-	-	0.025	-	-	-
Zygosaccharomyces bisporus	-	0.1	-	-	0.025	-	-	-
Cryptococcus saitoi	-	0.05	-	-	0.025	-	-	-
Candida quercitrusa	-	0.1	-	-	0.025	-	-	-
Filobasidium uniguttulatum	-	0.1	-	-	0.025	-	-	-
Percentage of isolates inhibited	**20%**	**100%**	**15%**	**0%**	**100%**	**0%**	**0%**	**10%**

-　　= no inhibition
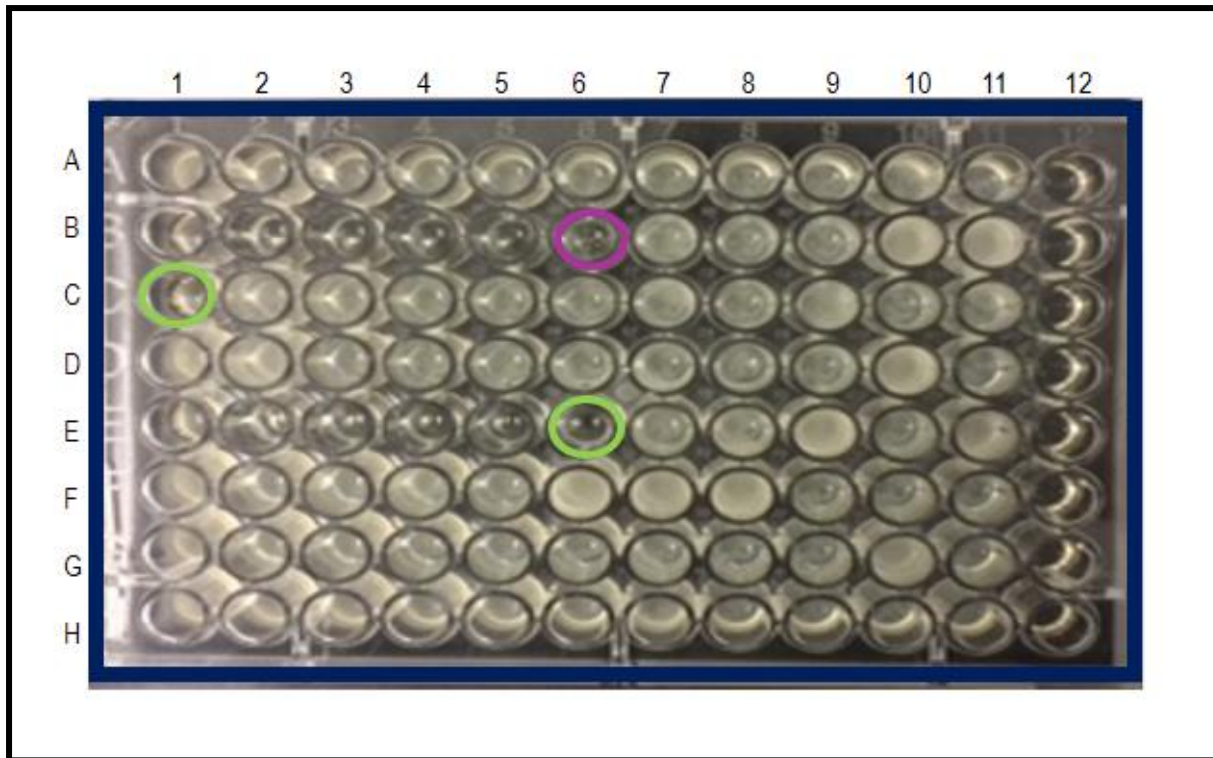
Figure 3.4: Representative 96 well plate demonstrating MIC results for *Wickerhamomyces anomalus*. Inhibition can be observed in wells B6 (disinfectant B at 0.05%), C1 (disinfectant C at 0.8%) and E6 (disinfectant E at 0.025%). The remaining wells shows no inhibition for disinfectants A, D, F, G and H. Rows 11 and 12 were the positive and the negative controls, respectively.

QAC's (disinfectants B and E) have been shown to have antimicrobial activity. Certain QAC's especially those containing long alkyl chains, are used as antimicrobials and disinfectants. Examples are benzalkonium chloride, benzethonium chloride, methylbenzethonium chloride, cetalkonium chloride, cetylpyridinium chloride, cetrimonium, cetrimide, dofanium chloride, tetraethylammonium bromide, didecyldimethylammonium chloride and domiphen bromide. (Carmano-Ribeiro & Carrasco, 2013).

In addition to having antimicrobial properties, QAC's are also excellent for hard-surface cleaning and deodorization. QAC's are membrane active agents i.e.*,* with a target site predominantly at the cytoplasmic (inner) membrane in bacteria or the plasma membrane which are anionic in yeasts (Lipke & Ovalle, 1998). The following sequence of events occur with microorganisms exposed to cationic agents: (1) adsorption and penetration of

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the agent into the cell wall; (2) reaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization; (3) leakage of intracellular low-molecular-weight material; (4) degradation of proteins and nucleic acids; and (5) wall lysis caused by autolytic enzymes. The cationic agents interact with phospholipid components in the cytoplasmic membrane, thereby producing membrane distortion and protoplast lysis under osmotic stress (Carmano-Ribeiro & Carrasco, 2013).

Reasons as to why disinfectant E works at lower concentrations than B, even though both are QAC's, can in all likelihood be due to the fact that the concentration of the active ingredient didecyldimethylammonium chloride is higher in the disinfectant E (0.12%) compared to Disinfectant B (0.02%) as per the material and product data sheets available for these disinfectants.

The mode of action of disinfectants A, C & D is oxidation related. The inhibition results of disinfectant A showed inhibition of four isolates (20%) and disinfectant D showed no inhibition at all. Aerobic organisms use molecular oxygen (O_2) for respiration or oxidation of nutrients to obtain energy. Reactive by-products of oxygen, such as superoxide anion radical (O_2-), hydrogen peroxide (H_2O_2), and the highly reactive hydroxyl radicals (OH), are generated continuously in cells grown aerobically. Most of such products are derive from sequential univalent reductions of molecular oxygen catalyzed by several membrane–associated respiratory chain enzymes. Environmental agents such as ionizing, near- UV radiation or numerous compounds that generate intracellular O_2 can cause oxidative stress, which arises when the concentration of active oxygen increases to a level that exceeds the cell's defence capacity (Cabisol *et al*., 1999).

The biological targets for these highly reactive oxygen species are DNA, RNA, proteins and lipids. Much of the damage is caused by hydroxyl radicals generated from H_2O_2 via the fenton reaction. Lipids are major targets during oxidative stress. Free radicals can attack directly polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters

membrane properties and can disrupt membrane-bound proteins significantly. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins (Cabisol *et al.*, 1999).

Disinfectants, at a high concentration, cause massive cellular damage at a macromolecular level, with different mechanisms of action depending on the chemical nature of the disinfectant. Oxidative disinfectants, such as chlorine and hydrogen peroxide (H_2O_2) remove electrons from susceptible chemical groups, oxidizing them, and become themselves reduced in the process (Winniczuk & Parish, 1997). At a cellular level, low levels of oxidation can be a highly reversible process and prokaryotic organisms have evolved many defences against these effects (Finnegan *et al.*, 2010).

At higher concentrations, these defence mechanisms can be overcome, with significant surface, cell wall and intracellular damage. Oxidizing agents are usually low-molecular-weight compounds and are considered to pass easily through cell walls/membranes, whereupon they are able to react with internal cellular components, leading to apoptotic and necrotic cell death (Winniczuk & Parish, 1997). Alternatively, they can severely damage microbial structure causing the release of intracellular components, which are then oxidized. The concentration exponent (h) of oxidizing agents is found to be in the low (<2) group suggesting that they interact strongly with their target by chemical, and not physical, means (McDonald & Russell, 1999). Although biochemical mechanisms of action may differ between oxidative biocides, the physiological actions are largely similar. Oxidative biocides are proposed to have multiple targets within a cell as well as in almost every biomolecule; these include peroxidation and disruption of membrane layers, oxidation of oxygen scavengers and thiol groups, enzyme inhibition, oxidation of nucleosides, impaired energy production, disruption of protein synthesis and, ultimately, cell death (Finnegan *et al.*, 2010).

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Oxidizing agents have been thought to react strongly with thiol groups in enzymes and proteins, DNA and the cell membrane. The redox potentials of the biocides used in a previous study differed, with peracetic acid having the highest, H_2O_2 next and chlorine dioxide (ClO_2) the lowest (Maris, 1995). Higher redox potentials indicate a greater tendency to acquire electrons and thus be reduced, with the electron donor species being oxidized, so one would expect that the substance with the highest redox potential would be the most effective oxidant (Maris, 1995). This was reflected in the oxidation of amino acids by these liquid agents, where peracetic acid produced the most oxidation and ClO_2 the least (Finnegan *et al*., 2010). Results indicate why disinfectant A (peracetic acid & H_2O_2) showed inhibition to 20% of isolates as compared to disinfectant D (ClO_2) showing 0% inhibition. The reason as to why other isolates were perhaps not inhibited by disinfectant A could be due to the concentration not being high enough to effect those particular isolates.

The disinfectants which modes of action are related to oxidation (A, C and D) displayed very little inhibition results (15, 20 and 0%). This could be due to the fact that the concentrations used were just not high enough to cause effective inhibition, as previously mentioned, at lower concentrations and low levels of oxidation can be an easily reversible process (Finnegan *et al*., 2010). These disinfectants showing very low levels of inhibition have similar modes of action to the current disinfectant (A) in use at the processing facility from which the isolates were also obtained. These isolates have been exposed for a very long time to this disinfectant and may have already adapted defence towards it and other disinfectants with similar modes of action (Trinneta *et al*., 2017). This could explain why oxidative form of attack was highly ineffective. Yeasts have been shown to have developed defence against oxidative stress. In certain yeasts, synthesis of catalase, superoxide dismutases, glutathione peroxidase, and a small protein of unknown function are induced in response to oxidative stress. Though the modes of action of oxidation have been shown to be devastating and cause cell death, mechanisms of defence have also been displayed by either reversing the oxidation

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process at low concentrations or producing agents in defence to the attack (Jamieson, 1992).

Modes of action such as irreversible binding to phospholipids and proteins of the membrane and oxidation, the irreversible binding to membrane components, have been clearly demonstrated to be more effective where defence mechanisms are not present versus oxidation where defence mechanisms are present and have been previously demonstrated. Not much detail on defence mechanisms are currently available in literature (Jamieson, 1992).

Disinfectants F, G and H are essential oils which has a mode of action of altering cell membrane structure and inhibiting respiratory enzymes. Essential oils have been recently used as disinfectants in the food industry for disinfection and control of spoilage, and studies have been conducted on antimicrobial activities of essentials oils against mostly bacteria, it has been found that Gram-negative bacteria are shown to be less susceptible than Gram-positive bacteria to antimicrobial effects of essential oils (Ioannou *et al*., 2006). This phenomenon is probably linked to the fact that the outer membrane of Gram-negative bacteria, which is composed of hydrophilic lipopolysaccharides, creates a barrier between the cytoplasmic membrane and hydrophobic compounds, such as essential oils, to protect the cell from antimicrobial effects (Trinetta *et al*., 2017).

The ability of the essential oils to inhibit the growth of yeast has also been previously evaluated and it was found that essential oils are not very effective against *S. cerevisiae* and *C. albicans*, as compared to Gram-positive bacteria. The specific cell membrane structure of yeast forms a barrier against various proteinaceous and non-proteinaceous molecules, therefore less sensitivity is observed (Trinetta *et al*., 2017). An additional reason which may also support the factor of inability of the tested essential oils to inhibit the yeast could be attributed to the recommended contact time of 30 minutes (Sola & Wirtanen, 2005). This however was not evaluated due to the fact that such prolonged periods of contact time required for disinfection would not be feasable in an production

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facility, where time lost would equate to less final product produced and thus reduced financial benefits (Maris, 1995).

The point of cost is of great significance to food production facilities for obvious reasons. The process of disinfection is seen as a cost impact to a business; however it is very important to protect product quality and the brand by avoiding recalls and spoilage incidences and providing safe product for human consumption. The disinfestation process has numerous impacts on a business such as decreasing efficiency and specific requirements for storage due to the chemical nature. The most important factor amongst these is the cost of disinfectants. When comparing the cost-in-use of the most effective disinfectant (E) against the currently used disinfectant (A), the most effective disinfectant will result in a cost saving initiative for the facility; R 89 316.00 per annum (Table 3.5). There is possibility however that disinfectant E may require additional water for the rinsing process as it is a high foaming agent. This will be determined by a facility trial.

Table 3.5: Cost comparison of most effective disinfectants versus that currently in use by the beverage facility in this study

Disinfectant	Recommended concentration	MIC effective (%)	Average monthly usage	Monthly cost	Annual cost
A (Current)	0.3 – 0.5	0.8	375 L	R 12 045,00	R 144 540,00
B	0.5 - 0.1	0.2	188 L	R 4 602,00	R 55 224,00
E	0.02	0.05	47 L	R 3 525,00	R 42 300,00

3.4 Conclusion

It is important to take all the possible contaminant(s) into consideration when choosing a disinfectant. Furthermore it is important to ensure proper and frequent monitoring of the more detailed information (i.e. getting the microbial information up to species level) is preferable. That way testing a new disinfectant before use would be possible. In the case where any fermentation which occurs in final products in the facility or growth is obtained from routine hygiene monitoring such as surface swabs, these cultures should be retained by means of cryopreservation of plate colonies and identified to determine if further resistance to a different disinfectant has occurred or a new species has been found to be causing spoilage or able to survive after disinfection has taken place.

Based on the yeast diversity and MIC results it can be derived that the disinfectant currently in use (A), with a peracetic acid active ingredient is not sufficient for this facility and switching to (E) with a $DDAC_2$ active ingredient, could have a significant impact on the inhibition of the survival of yeasts associated with the facility environment and product. Not only in choosing which disinfectant, but also the lowest possible effective dose. The possible cost saving is also noteworthy. This confirmation can be done by conducting a facility trial with usage of the disinfectants which performed most effectively in the MIC test to evaluate if disinfectants perform in the actual facility environment as effectively as they have on a lab scale test.

3.5 References

Amaro, R.L. & Nicaud, J.M. (2015). *Yarrowia lipolytica* as a biotechnological chassis to produce usual and unusual fatty acids. *Progress in lipid research* **61,** 40–50.

Aneja, K.R., Dhiman, R., Aggarwal, N.K. & Aneja, A. (2014). Emerging preservation techniques for controlling spoilage and pathogenic microorganisms in fruit juices. *International journal of Microbiology,* Article ID 758942, 14 pages.

Banerjee, P., Haider, M., Trehan, V., Mishra, B., Thakur, A., Dogra, V. & Loomba, P. (2013). *Crytococcus laurentii* fangemia. *Indian journal of medical microbiology* **31**, 75-77.

Barata, A., Malfeito-Ferreira, M. & Loureiro, V. (2011). The microbial ecology of wine grape berries. *International journal of food microbiology* **153**, 243-259.

Beuchat, L.R. (1987). *Food and Beverage mycology* 2nd edn Springer Dordrecht Heidelberg, London: United Kingdom.

Boulton, C. & Quain, D. (2001). *Brewing yeast and fermentation.* 1st edn John Wiley, London: United Kingdom.

Cabiscol, E., Tamarit. J, & Ros, J. (1999). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International microbiology* **3,** 3-8.

Carmona-Ribeiro, A.M. & Carrasco, L.D.D.M. (2013). Cationic antimicrobial polymers and their assemblies**,** *International journal of molecular sciences* **14,** 9906-9946.

Carolina Valeriano, C., de Oliveira, T.L.C., de Carvalha, S.M., Cardoso, M.D.G., Alves, E. & Piccoli, R.H. (2012). The sanitizing action of essential oil-based solutions against *Salmonella enterica* serotype *Enteritidis* S64 biofilm formation on AISI 304 stainless steel. *Food control* **25,** 673–677.

Coetzee, G., Malandra, L., Wolfaardt, G.M. & Viljoen-Bloom, M. (2004). Dynamics of a microbial biofilm in a rotating biological contactor for the treatment of winery effluent. *Water SA* **30**, 406-412.

Corbett, K.M. (2017). Diversity profiling and rapid detection of spoilage yeasts in a typical fruit juice bottling factory. [Dissertation]. Central University of Technology, Free State

Deak, T. (1991). Foodborne yeast. *Advanced applied microbiology 36***,** 123-127.

Ejdys, E., Biedunkiewicz, A., Dynowska, M. & Sucharzewska, E. (2013). Snow in the city as a spore bank of potentially pathogenic fungi. *Science of the total environment* **42,** 646-650.

Farrugia, G. & Balzan, R. (2012). Oxidative stress and programmed cell death in yeast. *Frontiers in oncology* **2,** 1-18.

Fickers, P., Benetti, P.H., Wache, Y., Marty, A., Mauersberger, S., Smit. M.S. & Nicaud JM. (**2005)**.Hydrophobic substrate utilisation by the yeast *Yarrowia lipolytica*, and its potential applications. *FEMS yeast research*, **5,** 527-543

Fiechter, A. & Seghezzi, W. (1992). Regulation of glucose metabolism in growing yeast cells. *Journal of biotechnology*. **27**, 27-45.

Finnegan, M., Linley, E., Denyer, S.P., McDonnell, G., Simons, C. & Maillard, J.Y. (2010). Mode of action of hydrogen peroxide and other oxidizing agents: differences between liquid and gas forms, *Journal of antimicrobial chemotherapy* **65**, 2108–2115.

Gadango, M. & Sampaio, P.J. (2001). Polyphasic taxonomy of the basidiomycetous yeast genus Rhodotorula: Rh. glutinis sensu stricto and Rh. dairenensis comb. Nov. *FEMS yeast research* **2,** 47-58.

Gerba, C.P. (2015). Quaternary Ammonium Biocides: Efficacy in Application. *Applied and environmental microbiology* **81**, *464-469.*

Guého, E., Smith, M.T., de Hoog, G.S., Billon-Grand, G., Christen, R. & Batenburg-van der Vegte, W.H. (1992). Contributions to a revision of the genus *Trichosporon.* Antonie Van Leeuwenhoek **61**, 289.

Hasejima, N., Kamei, K., Matsubayashi, M., Kawabi, R., Shimura, C., Hijikata, N., Oda, T. & Matsushima, H. (2011). The first case of bloodstream infection by *Candida intermedia* in Japan, the importance of molecular identification. *Journal of infection and chemotherapy* **17,** 555- 558.

Haupt, H.M., Beschorner, W.E., Vaughan, W.P. & Saral, R. (1983). Colonisation and infection of *Tricosporon* species in immunosuppressed host. *Journal of infectious diseases* **2,** 120-126.

Herskowitz, I. (1988). Life cycle of budding yeast *Sacchromyces cerevisiae. Microbiology reviews* **52,** 536-539.

Hinninger, W. & Windisch, S. (1976). *Torulopsis spandovensis* sp. nov. a new yeast from beer. *Archives microbiology* **104,** 529-535.

Ioannou, C.J., Hanlon, G.W. & Denyer, S.P. (2006). Action of disinfectant quaternary ammonium compounds against *Staphylococcus aureus* Antimicrobial agents and chemotheraphy **51**, 296–306.

Jamieson, D.J. (1992). *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. *Journal of bacteriology*, **174**, 6678-6681.

Keszthely, A., Hamari, Z., Pfeiffer, I., Vagvolgyi, C. & Kucsera, J. (2008). Comparison of killer toxin-producing and non-producing strains of *Filobasidium capsuligenum*: Proposal for two varieties. *Microbiological research* **163**, 267-276.

Kurtzman, C., Mateo, R.Q., Bart, A., Vincent, T. & Boekhout, T. (2015). Advances in yeast systematics and phylogeny and their use as predictors of biotechnologically important metabolic pathways. *FEMS yeast research* **15,** 890-907.

Lahlali, R., Serrhini, M.N. & Jijaki, M.H. (2004). Efficacy assessment of *Candida oleophila* (strain O) and *Pichia anomala* against major postharvest diseases of citrus fruits in Mexico. *Communications in agricultural and applied biological sciences* **69**, 601-610.

Levy, I., Rubin, L.G., Vasishtha, S., Tucci, V. & Sood, S.K. (1997). Emergence of *Candida parapsilosis* as the predominant species causing candidemia in children. *Clinical infectious diseases* **26,** 1086-1088.

Lin, D., Wu, L.C., Rinaldi, M.G. & Lehmann, P.F. (1995). Three distinct genotypes within *Candida parapsilosis* from clinical sources. *Journal of clinical microbiology* **33**, 1815–1821.

Lipke, P.N. & Ovalle, R. (1998). Cell wall architecture in yeast: New structure and new challenges. *Journal of bacteriology* **180,** 3735–3740.

Lund, B. & Baird-Parker, T.C. (2003). Fruit juices, Fruit drinks and Soft drinks. *Microbiological safety and quality of food* 1st edn Springer Dordrecht Heidelberg, London: United Kingdom.

Maris, P. (1995). Modes of action of disinfectants. *Revue scientifique et technique (international office of epizootics)* **14,** 47-55.

Mattsson, R., Haemig, P.D. & Olsen, B. (1999). Feral pigeons as carriers of *Cryptococcus laurentii, Cryptococcus uniguttulatus* and *Debaryomyces hansenii. Medical microbiology* **37**, 367-369.

Mazzola, P.G., Jozala, A.F., Novaes, L.C., Moriel, P. & Vessoni- Penna, T.C. (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents. *Brazilian journal of pharmaceutical sciences* **45**, 241-248.

McDonald, G. & Russell, A.G. (1999). Antiseptics and disinfectants: Activity, action, and resistance. *Clinical microbiology reviews* **12**, 147-179.

Men, L. & Wang, Y. (2007). The oxidation of yeast alcohol dehydrogenase-1 by hydrogen peroxide in vitro. *Journal of proteome research* **6**, 216-225.

Merico, A., Sulo, P., Piskur, J. & Compagno, C. (2007). Fermentative lifestyle in yeasts belonging to the *Saccharomyces* complex. *The FEBS journal*, **274**, 976–989.

Minegishi, H., Miura, T., Yoshida, Y., Usami, R & Abe. F. (2005). Phylogenic analysis from pectin degrading yeasts from deep sea environments. *Journal of Japanese society of extremophiles* **5,** 21-26.

Morgunov, I.G., Kamzolova, S.V. & Lunina, J.N. (2013). The citric acid production from raw glycerol by *Yarrowia lypolytica* yeast and its regulation. *Applied microbial biotechnology* **97**, 7387-7397.

Nakase, T., Suzuki, M., Takashima, M., Miyakawa, Y., Kagaya, K., Fukazawa, Y. & Komazata, K. (1994). *Candida sojae*, a new species of yeast isolated from the extraction process of water-soluble substances of defatted soy beans. *Journal of general applied microbiology* **40,** 161-169.

Nunes, J.M., Bizerra, F.C., Ferriera, R.C. & Colombo, A.L. (2012). Molecular identification, antifungal susceptibility profile, and biofilm formation of clinical and environmental *Rhodotorula* species isolates. *Antimicrobial agents and chemotherapy* **57,** 382-389.

Obasi, B.C., Whong, C.M.Z., Ado, S.A. & Abdullahi, I.O. (2014). Isolation and identification of yeast associated with fermented orange juice. *The international journal of engineering and science* **3***,* 64-69.

Oyamada, H., Ogawa, Y., Shibata, N., Okawa, Y., Suzuki, S. & Kobayahsi, H. (2008). Structural analysis of cell wall mannan of *Candida sojoe*, a new yeast isolated from defatted soy bean flakes. *Archives of microbiology* **5***,* 483-490.

Penna, T.C.V., Mazzola, P.G. & Martins, A.M.S. (2001). The efficacy of chemical agents in cleaning and disinfection programs. *BMC infectious diseases* **16,** 1-16.

Renker, C., Verena, B., Borstler, B., Heinrichs, J. & Buscot, F. (2004). Diversity of *Cryptococcus* and *Dioszegia* yeasts (Basidiomycota) inhabiting arbuscular mycorrhizal roots or spores. *FEMS yeast research* **4,** 597-603*.*

Rodrigues, M., Corte-Real, M., Leaou, C., Dijken, J.P.V. & Pronk, J.T. (2001). Oxygen requirements of the food spoilage yeast *Zygosaccharomyces bailii* in synthetic and complex media. *Applied and environmental microbiology* **67***,* 2123-2128.

Rodriquez, J.L.T., Barchiesi, F., Bille, J., Chryssanthou, E., Estrella, C.M., Denning, D., Donelly, J.P., Dupont, B., Fegeler, W., Moore, C., Richradson. M & Verweij, P.E. (2002). Method for the determination of minimum inhibitory concentration (MIC) by broth dilution of fermentative yeasts. *Clinical microbiology and infection* **9,** 1-8.

Rojo, C.C. & Rodriguez, C.O.C (2011). Importance of oxidative damage on the electron transport chain for the rational use of mitochondria-targeted antioxidants. *Mini reviews in medicinal chemistry* **7***,* 625-632.

Sabel, A., Martens, S., Petri, A., Konig, H. & Claus, H. (2014). *Wickerhamomyces anomalus* AS1: A new strain with potential to improve wine aroma. *Annals of microbiology* **64***,* 201-209.

Salo, S. & Wirtanen, G. (2005). Disinfectants efficacy on foodborne spoilage yeast strains. *Food and bio products processing* **83**, 288-296.

Schneider, J., Rupp, O., Trost, Y., Jaenicke, S., Passoth, V., Goesmann, A., Tauch, A. & Brinkrolf, K. (2012). Genome sequence of *Wickerhamomyces anomalus* DSM 6766 reveals genetic basis of biotechnologically important antimicrobial activities. *FEMS yeast research* **4,** 604-608*.*

Segal, R., Krtizman, A., Cadivalli, L., Samra, Z., David, M. & Tigva, P. (1996). Treatment of *Candida* nail infection with Terbinafine. *Journal of american academy of dermatology* **35,** 958-961.

Shankar, E.M., Kumarasamy, N., Bella, D., Renuka, S., Kownhar, H., Suniti, S., Rajan, R. & Rao, U.A. (2006). Pneumonia and pleural effusion due to *Cryptococcus laurentii* in a clinically proven case of AIDS. *Canadian respiratory journal* **5,** 275-278.

Simonin, H., Beney, L. & Gervais, P. (2007). Sequence of occurring damages in yeast plasma membrane during dehydration and rehydration: Mechanisms of cell death. *Biochimica et Biophysica Acta* **1768**, 1600-1610.

Stratford, M. (2006). The yeast handbook. Yeasts in food and beverages. In A. Querol & G. H. Fleet (Eds.), *Food and beverage spoilage yeasts* (pp 335-368). Berlin, Heidelberg: Springer-Verlag.

Subik, J., Kolarov, J. & Kovac, L. (1974). Anaerobic growth and formation of respiration-deficient mutants of various species of yeasts, *FEBS letter* **45**, 263-266.

Thomas, D.S. & Davenport, R.R. (1985). *Zygosaccharomyces bailii* – a profile of characteristics and spoilage activities. *Food microbiology* **2,** 157-169.

Trinetta, V., Morgan, M.T., Coupland, J.N. & Yucel, U. (2017). Essential oils against pathogen and spoilage microorganisms of fruit juices: Use of versatile antimicrobial delivery systems. *Journal of food science* **82**, 471-476.

Trofa, D., Gacser, A. & Nosanchuk, J.D. (2008). *Candida parapsilosis*, an emerging fungal pathogen. *Clinical microbiology reviews* **4**, 606-625.

Uscanga, B.A. & Francois, J.M. (2003). A study of the yeast cell wall composition and structure in response to growth conditions and mode of cultivation. *Letters in applied microbiology* **37,** 268-274.

Vohra, P. & Poxton, I.R. (2011). Efficacy of decontaminants and disinfectants against *Clostridium difficile. Journal of medical microbiology* **60**, 1218-1224.

Wang, Y., Wei, A. & Li, H. (2012) Using *Candida oleophila* as a biocontrol agents to prevent foodborne *Escherichia coli* O157 EHEC infections. 2nd edn Springerplus 1: 82. Published online 2012 Dec 28. doi: 10.1186/2193-1801-1-82.

Wessels, S. & Ingmer, H. (2013). Modes of action of three disinfectant active substances: A review. *Journal of regulatory toxicology and pharmacology* **67**, 456–467

Westblade, L.F., Rostad, C.A., Hilinski, J.A., Robert, T.S.C., Wilkey, J.K. & Pincus, D.H. (2015). *Candida quercitrusa* candidemia in a 6-year-old child. *Journal of clinical microbiology* **53**, 2785-2787.

Winniczuk, P.P. & Parish, M.E. (1997). Minimum inhibitory concentrations of antimicrobials against micro-organisms related to citrus juice. *Food microbiology* **14**, 373–381.

Winward, G.P., Avery, L.M., Stephenson, T. & Jefferson, B. (2008). Essential oils for the disinfection of grey water. *Water research* **42**, 2260–2268.

Xiao, M., Lu, H.W.J., Chen, S.C.A., Kong, F., Ma, X.J. & Xu, Y.C. (2014). Three clustered cases of candidemia caused by *Candida quercitrusa* and mycological characteristics of this novel species. *Journal of clinical microbiology* **52,** 1660-1668.

Yurkov, A.M., Kachalkin, A.V., Daniel, H.M., Groenewald, M., Libkind, D., de Garcia, V., Zalar, P., Gouliamova, D.E., Boekhout, T. & Begerow, D. (2015). Two yeast species *Cystobasidium psychroaquaticum* f.a. sp. nov. and *Cystobasidium rietchieii* f.a.

sp. nov. isolated from natural environments, and the transfer of *Rhodotorula minuta* clade members to the genus *Cystobasidium*. *Antonie Van Leeuwenhoek* **107**, 173–185.

Chapter 4

Selected disinfectants beverage

facility trial: CIP process and

molecular analysis

4.1 Introduction

The sanitation and disinfection of a process facility is one of the most critical aspects of food processing to ensure the health and safety of the consumer. Proper cleaning is essential for the production of high quality food products especially those with extended shelf life (Manning & Baines, 2004). Cleaning in place (CIP) is now a very common practice in many dairy, processed food, beverage and brewery plants replacing manual strip down, cleaning and rebuilding of process systems (Havelaar, 1994). The opposing method of disinfection is known as cleaning out of place (COP) (Tamine, 2009).

The definition of CIP is given in the 1990 edition of the Society of Dairy Technology manual "CIP: Cleaning in Place" as: "The cleaning of complete items of plant or pipeline circuits without dismantling or opening of the equipment, and with little or no manual involvement on the part of the operator (SPX, 2013). The process involves the jetting or spraying of surfaces or circulation of cleaning solutions through the plant under conditions of increased turbulence and flow velocity" (Niamsuwan *et al*., 2011).

CIP is not simply the provision of a CIP bulk unit, but the integrated process and hygienic design of the complete system (Tamine, 2009). A CIP system will consist of vessels for preparation and storage of cleaning chemicals, pumps and valves for circulation of the CIP chemicals throughout the plant, instrumentation to monitor the cleaning process and vessels to recover the chemicals (Thomas & Sathian, 2014).

An operator should routinely check chemical concentrations, pH levels, pump, and metering device performance. In addition, the water used in the CIP process should be monitored and verified because if the water used in the cleaning process is dirty, the system isn't able to clean pipes and tanks effectively (Thomas & Sathian, 2014). Similarly, the temperature of the cleaning solution should be monitored for complete control of the process (Niamsuwan *et al*., 2011). Last but not least, the disinfectant used is of utmost importance.

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Different factors also influence the effectiveness if the chosen disinfectant in use and these include: 1. Concentration: the presence of too little disinfectant will result in an inadequate reduction of harmful microorganisms. Too much can be toxic to humans. 2. Temperature: Generally disinfectants work best in water that is between 55℉ (13℃) and 120℉ (49℃). 3. Contact time: In order for the sanitizer to kill harmful microorganisms, the cleaned item must be in contact with the disinfectant for the recommended length of time (Fraser, 2003).

Many disinfectants are available in the market place for use in the food and beverage industry for the process of sanitation and disinfection (Sokunrotank *et al.,* 2012). The main categories of actions for disinfectants used in CIP processes are either oxidizing or non-oxidizing agents (Salo & Wirtanen, 2005).

The CIP process essentially has five steps which include, three rinsing steps, cleaning and disinfection and are shown in Figure 4.1 (Niamsuwan *et al.*, 2011). Not only should the CIP process be capable of removing soil and microbial contaminants, it should also have the ability to actively target areas where biofilm development is possible and if present, be able to destroy these biofilms (Fraser, 2003).

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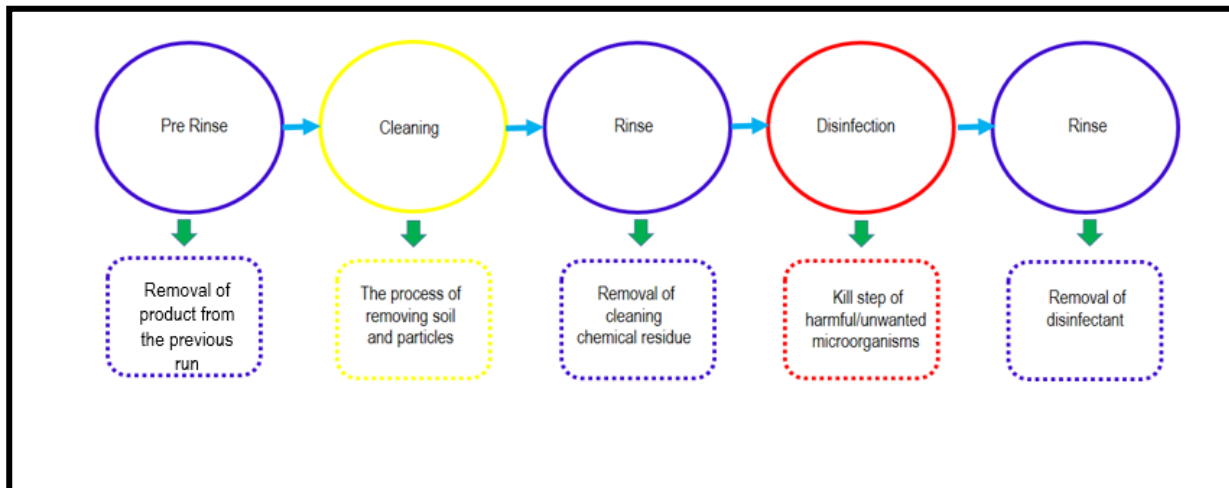
Figure 4.1: The five Step CIP process with description

Biofilms will form on almost any material where nutrients are available, but it happens more likely if the attachment surface is rough, scratched, cracked, or corroded. Physical conditions, such as hydrophobicity, surface electrostatic charge, and fluid flow rate also affect the attachment (Donlan, 2002). Several studies have shown that microorganisms attach more rapidly to hydrophobic, nonpolar surfaces such as Teflon (adhesive tape used in the food industry) and other plastics than to hydrophilic surfaces like stainless steel (Bridier *et al*., 2015). Some kind of hydrophobic interaction apparently occurs, which enables the cells to overcome the repulsive forces. Areas of interest where biofilm development can occur amongst others include filling machine nozzles in the beverage industry (Niamsuwan *et al*., 2011).

After subjection of effective disinfectants to the CIP process in a facility, further analysis can take place on equipment of interest to determine if disinfectants are indeed also capable of eliminating biofilms (Muyzer *et al.,* 1993). Such analysis can include not only microbial counts measuring the decrease which may take place in a trend analysis format, but also molecular analysis (Olsvik *et al.,* 1992). Molecular analysis includes analysis of DNA present to timeously identify organisms present. Identification can take place by genomic DNA extraction (Labuschagne & Albertyn, 2007), followed by PCR

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(Sharkey *et al*., 1994), and DGGE (Scorjetti *et al*., 2002) analysis and finally sequencing which will identify the organisms (Pavlov *et al*., 2004).

The aim of this study was to conduct a beverage facility trial of the two chosen most effective disinfectants from the MIC laboratory testing process (chapter 3) and determine true use effectiveness based on yeast enumeration and diversity analysis.

4.2 Materials and Methods

4.2.1 Fruit beverage facility

The participating facility decried in chapter three makes use of a CIP process. The blending and filling equipment, valves, pipework and nozzles are all of stainless steel construction. The process flow of the tanks and fillers start with feeding of raw materials from powder blenders (pre blend for transfer of powders and liquids) to blenders (final mixing equipment) where all the raw materials are combined in specific sequence stages to create a final concentrated beverage which is packed from filling tanks through nozzles into final production bottles before being capped. The equipment is shown in Figure 4.2.

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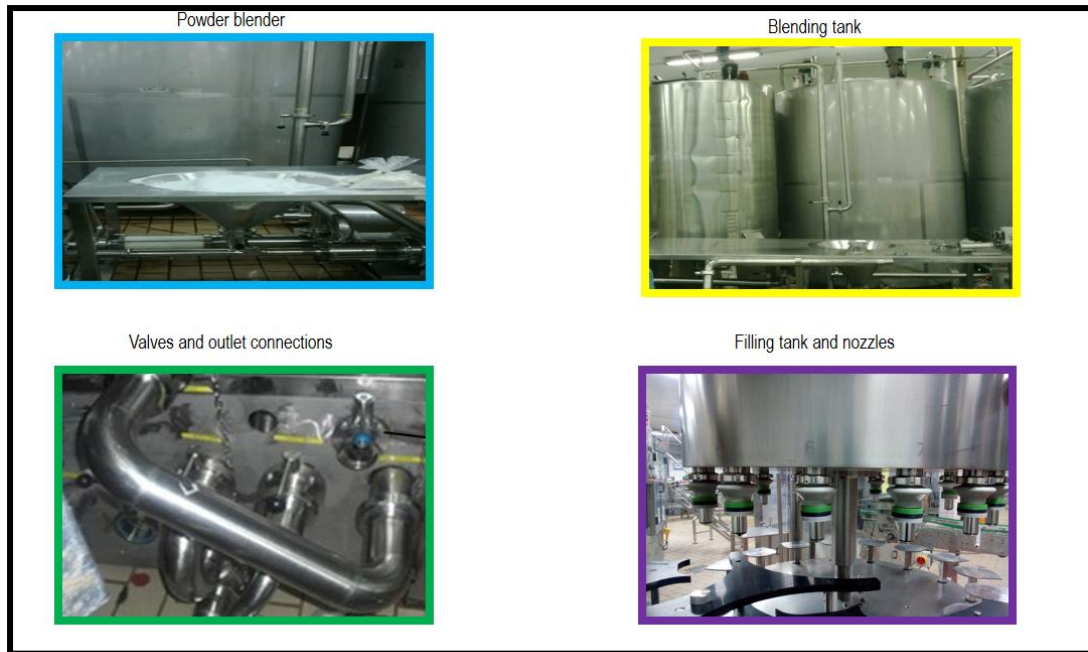
Figure 4.2: Stainless steel equipment used in the beverage bottling facility in this study

The facility is FSSC 22000 certified and has clearly defined "standard operating procedures" (SOP's) which describe all processes taking place within the organisation. FSSC 22000 certification is the second highest ranking of food safety certification within the industry currently. The process for CIP of the blenders and filling tanks are briefly described as per Figure 4.3. The CIP process is also extremely important not only for effective cleaning and disinfection to occur, but also as a process step known as O-PRP. The O-PRP is described as an operational prerequisite programme: Basic conditions and activities that are necessary to maintain a hygienic environment throughout the food chain suitable for the production, handling and provision of safe end products and safe food for human consumption, which in this facility's case, controls the possible contamination point of allergen as well as microbial contamination and growth (Hernández *et al*., 2018).
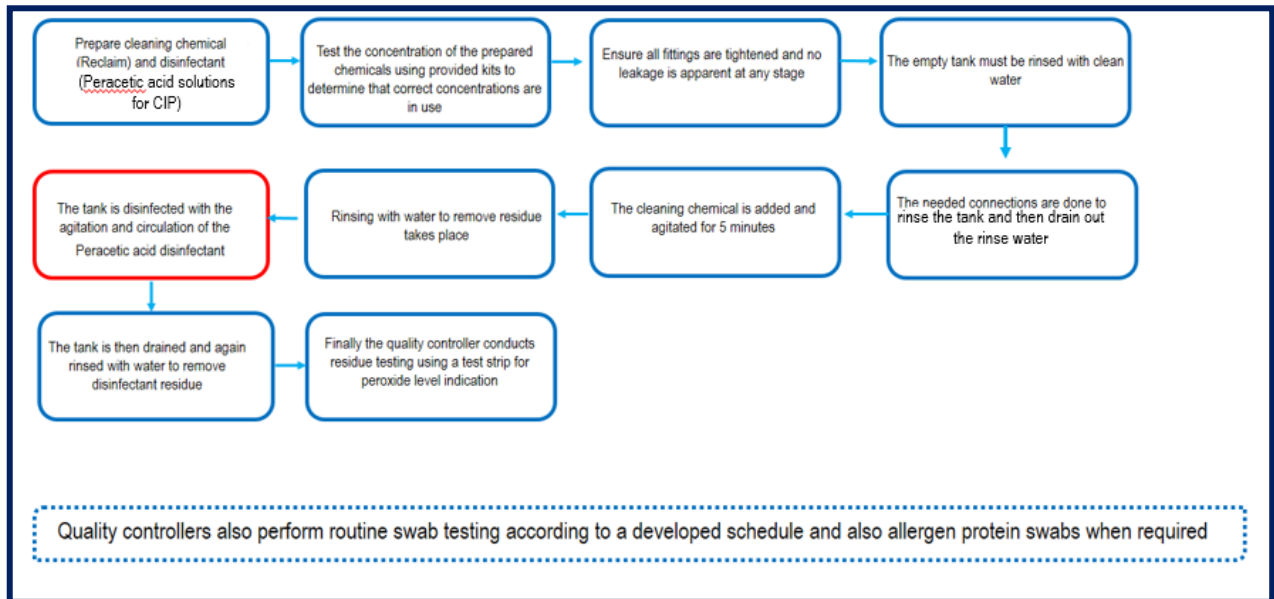
Figure 4.3: The CIP process with description used in the beverage facility in this study

The CIP step therefore is used as a two-fold purpose. Firstly CIP process facilitates cleaning and disinfection which is performed every 48 hours as per the recommendations of the current disinfectant supplier and secondly to conduct effective removal of previous product runs when moving within the production process from an allergen containing beverage to a non-allergen containing beverage

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4.2.2 Filling lines

The trial process utilized the two disinfectants (B & E) which displayed the best disinfectant capabilities from the MIC testing process in chapter three. The active ingredient in both disinfectants was didecyl dimethyl ammonium chloride (DDAC$_2$) and the only known difference between the two disinfectants was the concentration of this compound in the solution. The facility has five filling lines and two of the five were chosen to perform the trial. Processing line 1 (filler A) consists of 36 filling nozzles and processing line 2 (filler B) consists of 29 filling nozzles where the design slightly differed for the nozzles as shown in Figure 4.4.

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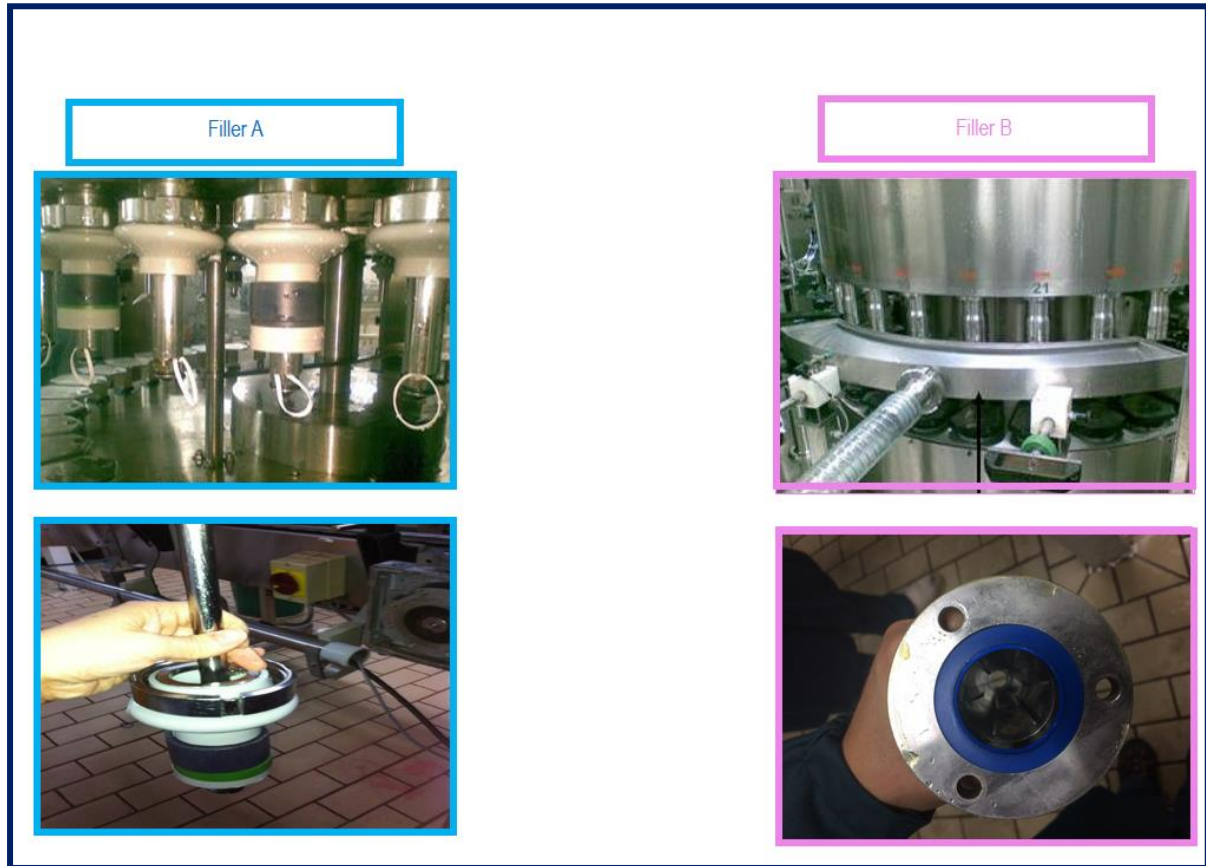
Figure 4.4: Nozzles of fillers A and B of filling machine in the facility in this study

4.2.3 Disinfectants utilized and CIP process

The CIP process used for the trial remained the same as the standard operating procedure used by the facility where the only difference was the disinfectant used and the verification strips used for the chemical concentration determination as well as residue testing after the CIP process. The fillers and blending tanks were both utilized in the trial CIP process and the usual schedule was maintained with 48 hour intervals as well as when allergen changeover took place. The residue and concentration testing strips differed from the current strips due to the active ingredient residue required to be tested as depicted in Figure 4.5.

Figure 4.5: Residue and concentration testing strips

For the concentration determination step the strips were utilized according to the manufacturer's instructions by dipping the test strip in the prepared solution and reading the colour difference which occurred. For residue testing after the CIP process the test strip was also dipped in rinse water from the filler or blending tank and the reading observed was 0 to indicate no residue was present. If the results indicated a reading other than 0, further rinsing would have to be performed.

4.2.4 The facility trial process

Disinfectant B was used at line 1 (Filler A) and disinfectant E was used at line 2 (Filler B). The process followed is summarised as follows: Before CIP, cleaning the lid of the manhole manually with a brush and VF8 (A general purpose cleaning soap) took place. The spray ball was removed and ensured to be clean (no foreign matter blocking the holes). The area around the motor shaft opening and top of the tank was also cleaned using a bottle brush and VF8 to prevent residues from having fallen into the tank. When the CIP was conducted it was ensured that all fittings were tightened and no water was leaking out of the fittings as the pressure in the pipe lines would have not been enough and the spray balls would not work effectively. When the tank was empty, it was rinsed with clean water to get rid of all the residue of the previous batch. The tank outlet was connected with the return back to the tank on the swing panel. All the water was then drained by disconnecting the swing bend and opening the needed valves. The tank was filled with 1000 liters water and an additional 5 liters (0.5%) of Reclaim (caustic for organic substance removal). The blender was switched on for 5 minutes of circulation after the concentration was first verified. While the tank was circulating Reclaim, the outside was washed with VF8 and a brush and rinsed with water. After 5 minutes the blender was stopped and drained. The tank was then rinsed out with water and then connected again to be filled with 1000 liters of water. Two liters of disinfectant B (Filler A) or 0.5 liters disinfectant E (Filler B) was added to the tank. The concentration was verified by quality controllers before circulation was again done for 5 minutes. Lastly, the tank was again drained and then rinsed with water and the final residue test was performed. When required as per SOP quality controllers also performed swab testing.

The chosen concentrations used were determined by taking the manufacturer recommendations together with the results of the MIC testing performed in chapter 3 and the contact time used was 5 minutes as per the current disinfectant used and not the manufacturer's recommendations (disinfectant E = 30 min suggested). The sampling events took place as per below for the two fillers:

- March 2017 (Time 0) for both fillers before changeover while using current disinfectant.
- Filler A on 22 March (Time 1) and 31 May 2017 (Time 2) and filler B on 24 March (Time 1) and 31 May 2017 (Time 2) after changeover to trial disinfectants.
- 25 September 2017 (Time 3) on both fillers. On 1 August a changeover back to the previously used disinfectant took place for the entire month. There after disinfectants B and E was again used for 3 weeks from 1 September, sampling was performed on 25 September.

4.2.5 Microbial sampling and analysis

Pre-trial hygiene swabs were taken where the CIP process took place as per the current SOP with current disinfectant. Swabs were taken of the nozzles after the CIP process where dismantling of the nozzles occurred. Swabs were then placed in YPD broth in a test tube and vortexed for 1 min. The YPD broth was plated onto MEA agar (spread plate technique) and incubated for 48 hours at 30°C followed by enumeration. Any growth was retrieved from plates by adding 1 ml of YPD containing 15% glycerol and spread across the plate with a hockey stick. The culture mixture was then collected with sterile pipette, transferred to cryogenic (cryo) vials and stored at -80°C until further analysis. The process was repeated after the changeover to the alternate two disinfectants as shown in Figure 4.6.
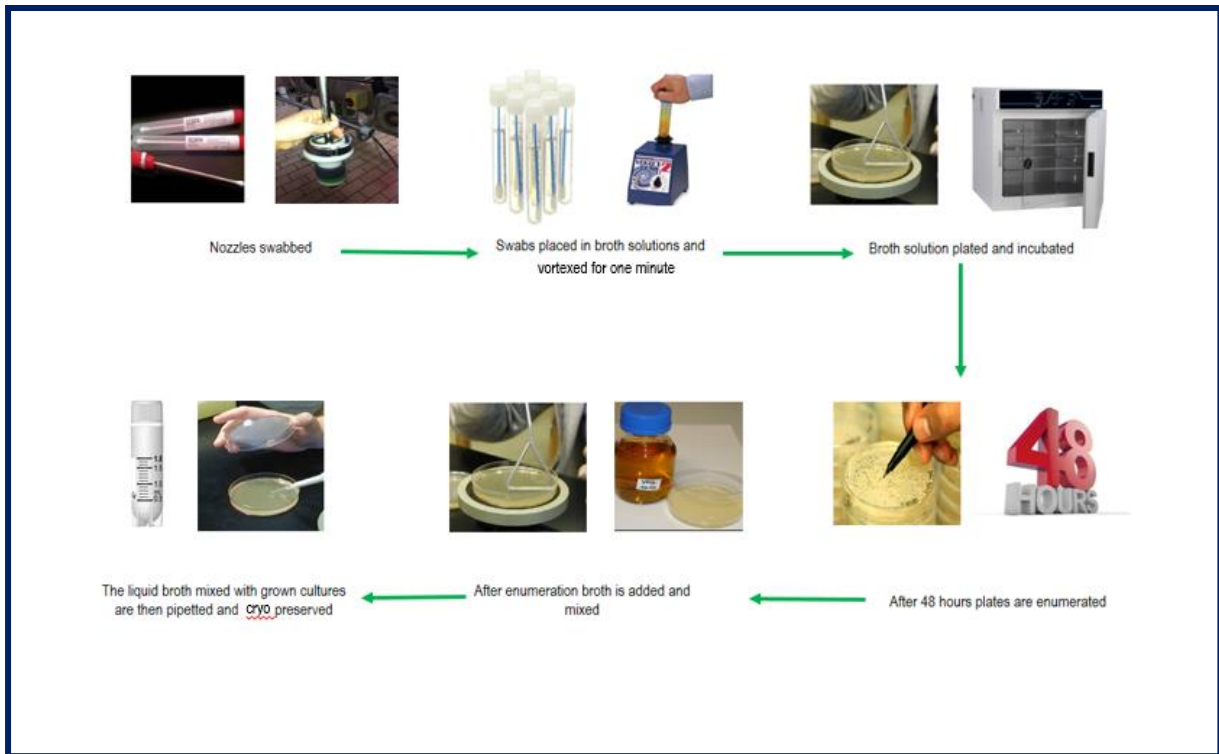
Figure 4.6: Microbial sampling process used in this study

The microbial monitoring conducted by the facility included total plate count as well as yeast and mould count using plate count agar (PCA) and chloramphenicol agar (CA), respectively. The equipment swabs were taken directly from equipment with no pre-soak in buffer required since the surfaces are already wet. The swabs were aseptically transferred to a test tube, vortexed, and plated accordingly. Differences in the standard versus the trial procedure can be seen in Figure 4.7
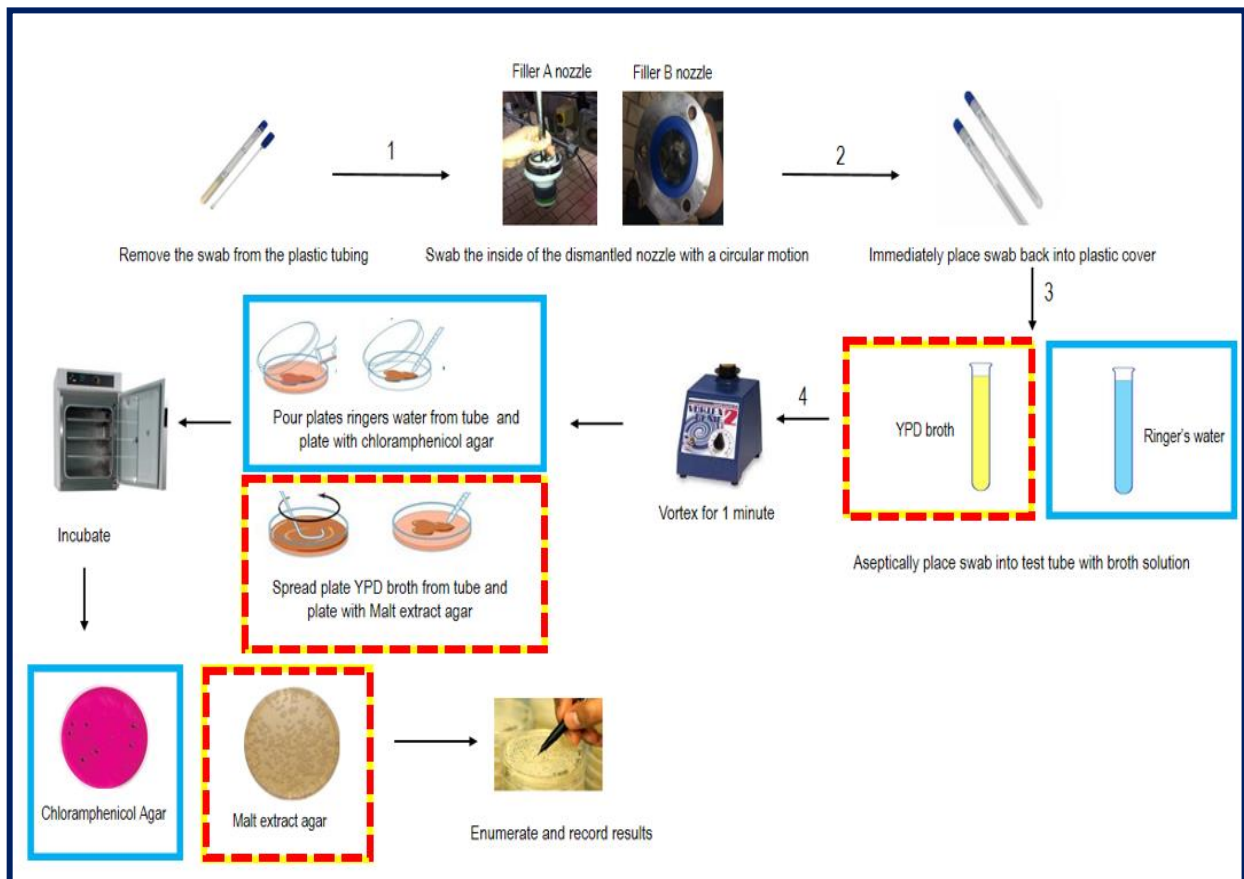
Figure 4.7: Differences in the SOP (blue) vs the trial process (yellow and red)

4.2.6 Equipment swabs

The facility conducts routine microbial monitoring of processing equipment such as powder blenders, blending tanks, pipelines, holding filler tanks, nozzles, and primary packaging materials. The equipment swabs are taken by trained quality control personnel following SOP's for sampling as well as microbial analysis. Swabs are taken once a week after the CIP process has taken place and in the case of a failure in results, the particular equipment would have undergone the CIP process again with immediate effect. The facility has a specification in place for the allowed amount of microorganisms which determine a satisfactory/unsatisfactory and failure result. These criteria can be seen in Table 4.1**.**

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Table 4.1: Criteria for determination of swab results for yeast and mould counts used by the beverage facility in this study

Count (cfu/ml)		Rating	Abbreviation
TPC	Yeast and mould		
0-49	0 – 10	Satisfactory	S
50 – 80	11 – 40	Unsatisfactory	US
>80	>40	Fail	_

4.2.7 Heat maps

Enumeration data were converted to heat maps using the Excel function of conditional formatting in Microsoft program. This was done by inserting values of interest, selecting the conditional formatting on the home bar, then selecting the third colour scale option on the drop list. The values used consisted of the change in colony forming units counts between each sampling point.

The heat maps therefore contained three columns of data, where column one was Time 0 – Time 1, column two was Time 1 – Time 2, and column three was Time 2 – Time 3. The different types of changes are depicted in different colours on the heat maps allowed a great tool to determine time frames of interest for conducting further analysis based on important changes in microbial counts which took place. The colours shown for the different types of changes consisted of light red indicating a significantly large increase (>-20 000), green indicating a significantly large decrease (>20 000) blue indicating a decrease (>1000), pink indicating an increase (>-1000) and white where insignificant or no change took place (<1000). In order to make use of the heat maps analysis, the colony counts amounts where the original counts obtained were too many to count and >300 cfu/ml, the value of 30 000 was used.

4.2.8 Genomic DNA extraction

Genomic DNA was extracted from the cryopreserved mixes described in 4.2.5 using a glass bead and detergent extraction method. Samples were pooled per sampling event by adding 20 µl of cell suspension from every cryo vial (each representing the yeasts present in a specific nozzle at a specific time/sampling interval). Pooled samples were centrifuged at 7 000 rpm for 5 min at 4°C and the pellets used for gDNA extraction. Lysis buffer treatment occurred as well as exposure to glass beads, followed by ammonium acetate, incubation at different temperatures and chloroform exposure, followed by addition of isopropanol and washing with ice cold ethanol. Finally, the DNA pellet was dried and resuspension in TE RNase (Labuschagne & Albertyn, 2007). The genomic DNA was separated in a 0.8 % agarose gel stained with ethidium bromide and visualized under UV light using the Gel Doc™ XR documentation system (Bio-Rad).

4.2.9 PCR amplification

The internal transcribed spacer (ITS) region was amplified using primer set ITS1-F^{GC} (5'-TCA TTT AGA GGA AGT AA-3') and ITS2 (5'-GCT GCG TTC TTC ATC GAT GC -3') (Liu *et al.,* 2015). The forward primer was modified on the 5' end with a GC-clamp (CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G). PCR reactions (50 µl) contained 1 µl of genomic DNA, 1X Reaction buffer (15 mM MgCl$_2$, enhancers and stabilizers), 200 µM dNTPs, 0.52 µM of each primer and 1 unit of PCRBIO HiFi Polymerase (PCR BIOSYSTEMS) (Sharkey *et al*., 1994). Bands of $\approx$ 450 bp were amplified using the following reaction conditions: Initial denaturation at 95°C for 3 min, 35 cycles of denaturation at 95°C for 15 sec, annealing at 55°C for 15 sec and elongation at 72°C for 30 sec (Barlett & Sterling, 2003). Final elongation was performed at 72°C for 5 min (Pavlov *et al*., 2004). Successful amplification was confirmed by separating and visualising DNA in a 1.2 % agarose gel as previously described (de Smidt *et al.,* 2014).

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4.2.10 *Denaturing gradient gel electrophoresis (DGGE) and data analysis*

Denaturing gradient gel electrophoresis (DGGE) was carried out using the DCode™ universal mutation detection system (Bio-Rad). Sequence specific separation of 30 µl of each PCR product was performed in 7% (w/v) polyacrylamide (Acrylamide/Bis 37.5:1) gel in 1X TAE buffer containing a 40–60% linear denaturant gradient. The 100% denaturant solution contained 40% (v/v) deionized formamide and 7 M urea. Electrophoresis was performed with a constant voltage of 100 V at 60°C for 12 hours. Gels were stained with 0.05% GelStar® (Lonza) for 5 minutes and rinsed with ultra-pure water. DGGE images were captured on the molecular imager Gel Doc™ XR and patterns analyzed with the Discovery Series Quantity One® 4.31 1-D analysis imaging software (Bio-Rad). A 5% band intensity threshold was set for band selection.

Individual bands were matched accordingly to their positions in the gel based on a 1.5% position tolerance and peak areas used to determine intensities (Cocolin *et al.,* 2001; Julien *et al.,* 2008; de Smidt *et al*., 2014). Cluster analysis describing pattern similarities among different samples was performed using an unweighted pair-group method with an arithmetic mean algorithm (UPGMA) and dice coefficient (Martinez-Alonso *et al*., 2010).

Where possible, representatives of all band positions were excised from the gel on the dark reader (Clare Chemicals Research), incubated in 50 µl ultra-pure water at 60°C overnight and 5 µl used as template for re-amplification. Re-amplification was performed using the same primers (ITS1-F primer without the GC-clamp), reaction setup and conditions as previously described. Re-amplification products (4 µl) were used as templates for sequencing with primer ITS2.

4.2.11 Sequencing analysis

Sequencing was performed on the ABI Prism 3130 XL genetic analyzer using the Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems). DNA was precipitated with EDTA and ethanol. Sequence homology searches were completed with the Basic Local Alignment Search (BLAST) server of the National Centre for Biotechnology Information for comparison of a nucleotide query sequence against a nucleotide sequence database (megablast) (Julien *et al.,* 2008). Only similarities with a BLAST index of 97% and above were considered for identification (de Smidt *et al.,* 2014).

4.3 Results and Discussion

4.3.1 Yeast culturing

The results for the year when the trial took place according to the facility's trending did not show any specific cause for concern as seen in Tables 4.2 and 4.3. Filler A showed counts obtained only four times in the entire year with only two failed results of >50, and filler B showed counts 14 times in the year with no failed results. The tables summarize the counts obtained. The disinfectant utilized by the facility at this time was disinfectant A which was also included in the MIC test procedure (chapter 3).

Table 4.2: Annual facility microbial monitoring yeast results for filler B nozzle swabs

	Yeast counts cfu/ml were obtained from nozzle swabs after CIP Filler B											
Month	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
1	0	0	0	3	0	1	0	0	0	0	0	0
2	0	0	0	8	0	0	11	0	0	0	0	0
3	0	0	0	0	0	0	25	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	2	0	0	0	0	0	0	0
6	0	0	0	0	1	0	0	0	0	0	0	0
7	0	0	0	0	1	0	0	0	0	0	0	0
8	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	19	0	0	0	0	1	0	0	0	0
14	0	0	0	0	0	0	0	5	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0	0	0	0	0	0
17	2	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	1	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	1	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0

Nozzle number (row label, vertical)

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Table 4.3: Annual facility microbial monitoring yeast results for filler A nozzle swabs

Yeast counts cfu/ml were obtained from nozzle swabs after CIP Filler A												
Month	**Jan**	**Feb**	**Mar**	**Apr**	**May**	**Jun**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Dec**
1	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0	0	0
5	0	0	0	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0	0	0	0	0	0	0
7	0	0	0	0	0	0	0	0	0	0	0	0
8	0	0	0	0	0	0	15	0	0	0	0	0
9	0	0	0	0	0	0	0	1	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0
13	0	0	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	0	0	0	0	0
16	0	>50	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0	0	0	0	0
23	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	0	0	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0	0	0
26	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0	>50	0	0	0	0	0	0	0	0	0
34	0	0	0	0	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0	0	0	0	0

(Row label: Nozzle number)

The trial conducted was done with swabs taken before and after changeover to the two different disinfectants as seen in Table 4.4. The striking results even before changeover shows a vast difference when compared the facility's monthly swabbing results which rarely show any areas of concern where zero counts were obtained nearly always.

The nozzles in particular were the chosen points for the swabbing process due to the possibility of biofilm development as these nozzles are not dismantled during each and every CIP process. The first sampling event included every second nozzle of each filler which was dismantled and swabbed. The reason for every second nozzle was to avoid the disruption of possible biofilms which may have been present. The facility process of taking swabs of nozzles only included a once a month dismantling of nozzles which occurred, for weekly swabbing the nozzles were swabbed in place with no dismantling taking place.

Table 4.4: Results of the trial before and after changeover to different disinfectants and back to the previous disinfectant (A)

Nozzle	Disinfectant B (Filler A)				Disinfectant E (Filler B)			
	cfu/ml average of duplicate plates							
	Time 0	Time 1	Time 2	Time 3	Time 0	Time 1	Time 2	Time 3
1	TMTC	TMTC	0	-	6400	22000	15600	0
2	-	>300	0	0	-	400	200	2500
3	5050	>300	0	-	1600	6900	50	0
4	-	10400	0	0	-	16100	350	200
5	6600	750	0	-	9150	TMTC	0	0
6	-	>300	0	0	-	TMTC	4900	100
7	150	700	0	-	400	TMTC	300	200
8	-	0	0	100	-	5100	100	0
9	100	17000	0	-	>300	900	950	3000
10	-	14750	0	100	-	9100	700	0
11	0	150	0	-	8400	8200	400	0
12	-	300	0	200	-	9700	0	2400
13	850	3100	0	-	100	3050	300	0
14	-	15250	0	TMTC	-	3750	6650	0
15	0	0	0	-	500	15450	850	TMTC
16	-	2600	0	7000	-	300	0	TMTC
17	0	9150	0	-	3750	6500	600	0
18	-	26100	0	5600	-	12150	12550	0
19	2100	550	0	-	5650	2700	8950	1800
20	*	*	*	*	-	1900	13900	0
21	8200	0	0	-	350	12350	750	0
22	-	6850	7650	0	-	700	17850	8000
23	Mould	800	0	-	1550	4100	1400	0
24	-	9250	0	100	-	>300	500	500
25	0	8100	0	-	7350	TMTC	200	2600
26	-	1000	0	3000	-	5250	2650	0
27	4300	2650	2500	-	1650	26100	100	5500
28	-	500	0	600	-	7250	350	200
29	100	>300	0	-	-	5400	0	*
30	-	1350	0	1000	N/A	N/A	N/A	N/A
31	0	0	0	-	N/A	N/A	N/A	N/A
32	-	4300	0	4000	N/A	N/A	N/A	N/A
33	-	>300	0	-	N/A	N/A	N/A	N/A
34	-	6150	0	400	N/A	N/A	N/A	N/A
35	3300	650	0	-	N/A	N/A	N/A	N/A
36	-	TMTC	0	400	N/A	N/A	N/A	N/A

* = Blocked out nozzle, not in use
- = Nozzle not swabbed at the time
TMTC = Too many colonies to count
N/A = Not applicable
Time 0 = 06 March 2017 Old disinfectant
Time 1 = 22 March 2017 (filler A) & 24 March 2017 (Filler B) New disinfectant
Time 2 = 31 May 2017 New disinfectant
Time 3 = 25 September 2017 reverted to disinfectant used previously for the month of August and then back to trial

Disinfectant E performed rather differently than expected where reduction by 100% in counts only occurred by a percentile of 13 (4 out of 29 nozzles) in the same two month period. A further note observed was that after initial swabs the counts on nozzle swabs increased in certain instances for both disinfectants. The theory behind this could possibly be the disruption of biofilms which may have been present at the time and were in the process of disruption resulting in a large number of free cells which increased the plate count on the yeasts colonies.

The process of biofilm disruption occurs resulting in the cells being released and then becoming susceptible to disinfectants. Previous experimentation indicates this process which occurs meaning that the cells within the biofilm will be released and not immediately destroyed or inhibited, this process may then still need to take place, which conceivably explains an increase in the number of colonies counted on plates before the single cells were able to be destroyed or inhibited by the disinfectants during the disruption and detachment process (Brockson *et al*., 2014).

Detachment refers to the release of cells or clusters from the surface of the biofilm into the bulk fluid. Several factors can contribute to detachment, including matrix-degrading enzymes, nutrient levels, and quorum-sensing signals. When detachment occurs the biofilm desrupts and free cells are then released (Jang *et al*., 2017). The detachment of the biofilm and release of cells can either be caused by life cycle itself or in this case an inhibiting agent which is this case would be the disinfectants (Brockson *et al*., 2014).

In the case of disinfecant E, the yeast counts still increased even further by 17 percent (5 out of 29 nozzles) during the trial process instead of the swift decrease in the case of disinfecant B. Two months after the changeovertook place, there were still some nozzles showing an increase in yeast counts instead of a decrease. This could possibly be attributed to the fact that the disinfectant action was much slower than compared to MIC testing, and a shorter contact time than recommended being used (Mazzola *et al.,* 2009).

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A factor which was noted is the difference between the nozzles of the two fillers. Filler A nozzles were very easily removed for the swabbing with just a clockwise unscrewing motion which took only a couple of seconds, whereas filler B nozzles are fitted much more tightly, making removal/opening for swabbing much more time consuming, where unscrewing of each nozzle required removal of four fitted screws which took anywhere from 5 to 10 minutes to complete. During swabbing it was also noted, on rare occasions, that some final product from the run prior to the CIP still remained on the nozzles, which is directly screwed against the bottom of the filler unit. This indicates that the nozzles of filler B are more complex and difficult to reach when compared to filler A nozzles. This difference could also be the reason for the "slower" action/performance of disinfecant E where the bioflims and yeast in general present in the nozzles of ffiller B are at greater quanities making additional "work" required to be performed by disinfectant E.

The excellent performance shown in the trial process point toward disinfectant B as the preferred choice to reduce the yeast load present in the vital areas within the facility's equipment to practically 0. The reasons behind why disinfectant B performed better than disinfectant E could also be due to the concentrations used. These concentrations were chosen according to the MIC test results together with manufacturer's recommendations. Where disinfectant B was used at a concentration of 0.2%, disinfectant E was used at 0.05%. The contact time recommended by the manufacturer for disinfectant E of 30 minutes was a time which the facility would just not allow as it would result in major production time losses. The facility trial was still conducted with a 5 minutes contact time for both disinfectants as both showed the ability to inhibit the growth of the tested yeast in this time span during the MIC testing.

Another factor possibly impacting the performance of disinfectant E could be that it was able to cause inhibition at low concentrations in a lab scale on a single isolate at a certain concentration (Araujo *et al*., 2013). However, this effect was not replicated when used in facility scale trials due to many different isolates present or in a biofilm environment and a shorter than recommended contact time (Theraud *et al*., 2004).

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Also, various factors in the facility and environment which had an influence in contrast to a controlled MIC test environment such as equipment surfaces, water quality, possible remaining product residues and defence mechanism of multiple yeasts in complex biofilms (Theraud *et al.*, 2004).

There was also noted differences of the products packed on the different lines before and on swabbing dates where different types of products could have also impacted microbial load present in the filling tanks to which nozzles are attached before the CIP and swabbing took place. The different types of products packed are summarized in Table 4.5. The differences show that filler A packed only squashes preceding and on all swabbing dates, whereas filler B packed squashes, nectars and cordials.

Table 4.5: Different products packed before and on swabbing dates during the trial

Date of swabbing after CIP 2017	Product packed preceding CIP	Product packed on CIP date
	Filler A	
06 March	Squash	Squash
22 March	Squash	Squash
31 May	Squash	No production
25 September	Squash	No production
	Filler B	
06 March	Nectar	Nectar
24 March	Cordial	Squash
31 May	Squash	No production
25 September	Squash	No production

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The differences noted in these products are that squash and nectars contain both pulp as well as concentrate. Cordials contain neither pulp nor concentrate. The average pH of all products remains below 3.5 and other ingredients present on all produces include sweeteners, acids, flavourants, colourants, stabilizers, water, sugar and preservatives. Other characteristics noted which could influence microbial load are summarized in Table 4.6. The major differences to note would be the presence of pulp and concentrates, the major differences in ºBrix and acidity which of course greatly impact the favourable growth of yeasts in nectars more than squashes and cordials (Deak, 1991).

Table 4.6: Differences in Squash, Nectar and Cordial drinks

Product	Pulp	Concentrate	Average º Brix	Average acidity g/l
Squash	Yes	Yes	13.5	15.1
Nectar	Yes	Yes	44.5	19.5
Cordial	No	No	2.1	8.5

The last concern before the commencement of the trial was the known disadvantage of both disinfectants in that they are high foaming agents, which then led to the belief that additional water would be a prerequisite to rinse in the final step of disinfection to rid all foam which would have developed. However, this was not the case. The trial process used the same amount of water which was usually required in the final rinse step. A need for additional water would of course not be welcomed in a production environment where water saving is considered as a top priority and increases processing cost (additional water as well as time) and therefore decreases profitability.

The signicant changes in the microbial counts which took place during the four different sampling events is fittingly visible in the heat maps constructed and presented in Figure 4.8. The heat maps clearly portray the changes in microbial counts which took place throughout the four different sampling events as per the different colour schemes. The maps also again portray the effectiveness of disinfectant B versus disinfectant E where

significant decreases are much more for disinfecant B than E after changeover took place. The dark green and blue colours are of interest as they indicate signicant decrease in counts between two sampling events. The red and pink colours in cells are likewise of interest as these show signicant and small increases in counts observed, which of course is the opposite intent of the trial results anticipated although for the first sampling event it can be explained by disruption of bioflims taking place.

Between sampling event zero and one, disinfectant B showed signicant and well as slight increases in counts observed in six nozzles. Decreases in counts were noted in five nozzles. The most striking changes for disinfectant B took place between sampling events two and three. Where significant and slight decreses were noted in 13 and five nozzles respectively showing a positive impact of the disinfectant in action against the yeasts present in the nozzles. In many instances the significant decresed which took place are equavalent to 3 or 4 log reductions in counts which futher reveals the effectiveness of this disinfectant to act against the yeast present within the bioflim enviroment in a time frame of two months after continious use.

Filler A			
Nozzle	Time 1-2	Time 2-3	Time 3-4
A1	0	30000	
A2		30000	0
A3	-24950	30000	
A4		10400	0
A5	5850	750	
A6		30000	0
A7	-550	700	
A8		0	-100
A9	-16900	17000	
A10		14750	-100
A11	-150	150	
A12		300	-200
A13	-2250	3100	
A14		15250	-30000
A15	0	0	
A16		2600	-7000
A17	-9150	9150	
A18		26100	-5600
A19	1550	550	
A21	8200	0	
A22		-800	7550
A23		800	
A24		9250	-100
A25	-8100	8100	
A26		1000	-3000
A27	1650	150	
A28		500	-600
A29	-29900	30000	
A30		1350	-1000
A31	0	0	
A32		4300	-4000
A33		30000	
A34		6100	-400
A35	2650	650	
A36		30000	-400

Filler B			
Nozzle	Time 1-2	Time 2-3	Time 3-4
B1	-15600	6400	15600
B2		200	-2300
B3	-5300	6850	50
B4		15750	150
B5	-20850	30000	0
B6		25100	4800
B7	-29600	29700	100
B8		5000	100
B9	29100	-50	-29050
B10		8400	700
B11	200	7800	400
B12		9700	-2400
B13	-2950	2750	300
B14		-2900	6650
B15	-14950	14600	-29150
B16		300	-30000
B17	-2750	5900	600
B18		-400	12550
B19	2950	-6250	7150
B20		-12000	13900
B21	-12000	11600	750
B22		-17150	9850
B23	-2550	2700	1400
B24		29500	0
B25	-22650	29800	-2400
B26		2600	2650
B27	-24450	26000	-5400
B28		6900	150
B29		5400	

Figure 4.8: Heat maps of disinfectant B and E based on the changes in yeast counts among the four sampling events.

(red)	Large increase
(green)	Large decrease
(blue)	Decrease
(magenta)	Increase
(white)	Hardly/no change

Sampling event differences of times three and four also depict again the log increases taking place when the facility reverts back to the disinfectant used previously (disinfectant A) for a month, which is clearly ineffective, and back to the trial disinfectant where yeast growth inhibition is again evident. Time three only had counts in two nozzles where reversion to the old disinfectant (A) and back to the trial already displays significant and slight increases in 13 nozzles and only a significant decrease in one nozzle.

Disinfectant E shows significant and slight increases in yeast growth during time one and two. Where increases here are seen in 11 nozzles and only one significant decrease is noted. Again as per disinfecant B, the sampling events of times two and three are of most interest where significant and slight decreases took place in 19 nozzles, however not to the extent of disinfectant B. The change to decreasing counts between sampling events is much slower using disnfectant E. Even significant increases were also still taking place between these two sampling events, which further depicts the less effective result of this disinfecant. Return to the old disinfectant and back to the trial showed significant and slight increases in many nozzles during the last two sampling events which again demonstrates the inabilitiy of the old disinfectant (A) to control yeast growth and the return of biofilm formation within a short time frame.

Overall, the efficacy of disinfectant B was highlighted by the changes seen between times two and three for with all the significant and slight decreases shown by the dark green and blue colours. The heat maps unmistakably show this outcome and further display the optimistic decreases in counts disinfectant B had accomplished.

4.3.2 Molecular analysis

To design adequate strategies to prevent spoilage, it is advantageous to not only know the number of yeasts present on the nozzles, but also their identities. Denaturing gradient gel electrophoresis (DGGE) was performed to investigate the effect of the different disinfectants on the culturable, biofilm associated yeast diversity in the nozzles

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of fillers A and B. Intact genomic DNA was extracted from pooled culture samples and a ≈ 450 bp fragment of the ITS region amplified using endpoint PCR (Figure 4.9).
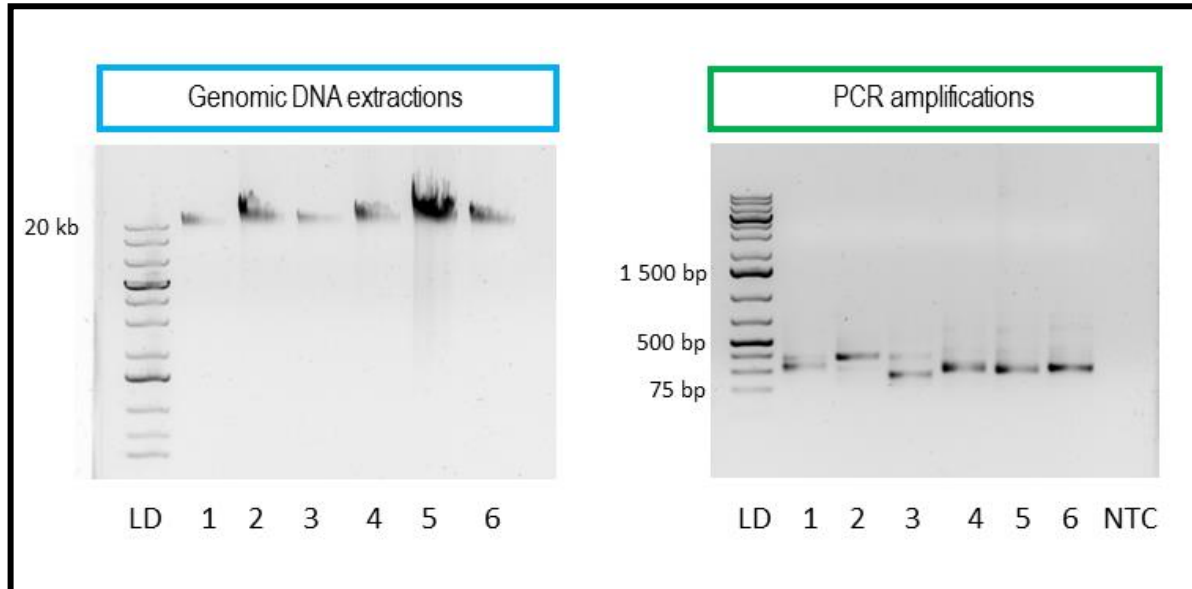

Figure 4.9: Agarose gels depicting gDNA (0.8%) and PCR products (1.2%) extracted and amplified from fillers A and B pooled samples during the facility trial. Lane LD contains GeneRuler™ 1kb DNA Ladder Plus (Thermo Scientific), lanes 1-3 samples A0-pooled, A1-pooled and A3-pooled, lanes 4-6 samples B0-pooled, B1-pooled and B3-pooled. Lane NTC represents a non-template PCR control.

PCR products were separated by DGGE to assess the yeast diversity. Figure 4.10A depicts the DGGE results and calculated cluster analysis. The dendrogram shows the differences which can be seen between both Filler A and B samples and the changes in yeast diversity during the trial process. Regrettably, the agar plates with yeast growth sampled at Time 2 (on 31 May 2017 for both fillers) were not cryo preserved due to a misunderstanding between the lab and the facility and does not form part of the molecular analysis data set. Efficacy of the disinfectants used was judged by a decrease in the number of bands present as well as in % similarity (Figure 4.10B). A 50.4% and 86.7% similarity was calculated between A0-pooled and A1-pooled, and B0-pooled and B1-pooled, respectively and only a 26.3% and 46.1% between sampling events 2 and 3 for fillers A and B respectively. The band positions obtained showed the change in yeast diversity that occurred during the trial process where certain bands remained throughout

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for both fillers A and B. There consistent presence demonstrated that neither disinfectants were able to eliminate the yeast species represented by those band positions, and some resurfaced after the switch back to disinfectant A.

Other bands appeared in A1-pooled and B1-pooled which were not present during the A0-pooled and B0-pooled sampling events. They could easily have been missed as only every second nozzle was swabbed during the first sampling event. The disappearances of bands in A1-pooled and B1-pooled indicated the disinfectants effectively eliminating the yeast species represented by that specific band position from the biofilm in the nozzles.
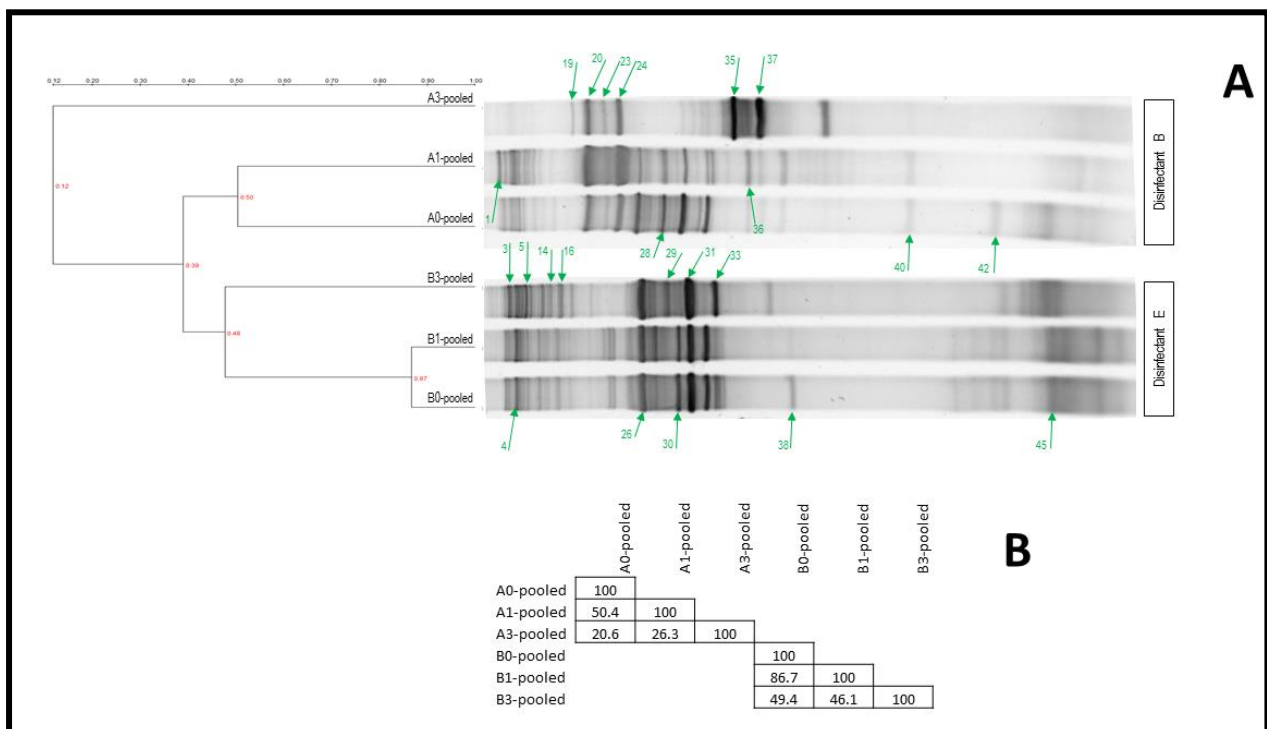

Figure 4.10: (A) UPGMA dendrogram representing cluster analysis of ITS region banding profiles of the nozzle swab pooled samples. The green numbers and arrows indicate assigned band positions that were excised and sequenced for identification. (B) Inter-sample similarities (%) demonstrated by Dice coefficient matrices.

A0-pooled & B0-pooled (Time 0) – Old disinfectant before changeover

A1-pooled & B1-pooled (Time 1) – 22 March 2017 (Filler A) & 24 March 2017 (Filler B) New disinfectant

A2-pooled & B2-pooled (Time 3) – 25 September 2017 reverted to old disinfectant for a month (August) and then back to trial

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The diversity obtained depicted a vast number of band positions, although a total of only seven different genera of yeasts were identified and one unknown isolate. Different band positions represented the same species in many cases, which is not ideal but, also not an uncommon characteristic in these types of analysis methods. The reason behind this occurrence has been found in previous studies as being the behaviour due to the different sources, causing different migration in the gels (Marzorati *et al.*, 2008). Table 4.7 provides the summary of the identification of the different band positions obtained and analyzed.

Table 4.7: Summary of sequences obtained from excised DGGE bands and the closest match from the Genbank database. The isolate descriptions are partial sequences of the 18S ribosomal RNA gene; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2. Different text colours indicate isolates classified under the same family.

Isolate	Band positions	E-value	Identity	Accession number	Base pairs
Candida sojae strain CBS 7871	1, 4, 26,	7.00E-106	99%	KJ722419.1	217
Lodderomyces elongisporus culture CBS:2605	14, 16, 31, 36, 38, 42, 45	7.00E-121	100%	KY104078.1	245
Kazachstania exigua culture CBS:379	3, 5, 20, 23, 24, 40	3.00E-109	100%	KY103631.1	219
Pichia occidentalis strain F028/*Candida inconspicua* isolate H137	37	9.00E-53/1.00E-51	100%	KY580388.1/ KU238836.1	155
Rhodotorula dairenensis culture CBS:4406	29, 33	9.00E-99	100%	KY104735.1	203
Sporidiobolus sp. FA-8H	28	7.00E-100	100%	JX164071.1	207
Rhynchogastrema noutii culture CBS:8365	19, 35	1.00E-67	97%	KY104935.1	166
Uncultured fungus clone 107A68238 1	30	3.00E-109	98%	JX334310.1	239

The yeasts could be classified under two phyla namely Ascomycota and Basidiomycota. Four Ascomycota species detected grouped under the Saccharomycetales order representing two families; Debaryomycetaceae (*Candida sojae* and *Lodderomyces elongisporus)* and Saccharomycetaceae (*Kazachstania exigua* and *Pichia occidentalis)*. Basidiomycota species could be classified under Pucciniomycotina and Agaricomycotina sub phyla. The Pucciniomycotina subphylum contained isolates from the Microbiotryomycetes class, Sporidiobolales order and Sporidiobolaceae family (*Rhodotorula dairenensis* and *Sporidiobolus* sp.*)*. Lastly, in the Agaricomycotina sub phylum, Tremellomycetes class, Tremellales order and Rhynchogastremataceae family, *Rhynchogastrema noutii* was identified.

Candida sojae is a species which has been isolated from defatted soy beans flakes, plague insect in cane cultivar, sugar cane bagasse and in the tanning industry wastewater (Nakase *et al*., 1994). It has been found to be capable of biofilm formation (Borelli *et al*., 2016). This species shown in band position 1, 4 and 26 on the gel, showed presence in lane A1-pooled, but no presence in lanes A0-pooled and A2-pooled, showing disinfectant B being able to rid this organism. However, B0-, B1- and B2-pooled all showed the presence of this yeast in numerous band positions demonstrating that it was present in abundance from the beginning in the nozzles of filler B and was unable to be removed during the trial process or when reverting to previous disinfectant. *Candida sojae* was, therefore, possibly resistant/tolerant to low concentrations of the active ingredient in disinfectant E.

Lodderomyces elongisporus has been isolated in soft drinks and juice factory environments, cocoa, soil, human fingernail, human blood and baby creams (Deak 1991; Kurtzman, 2003). It has been shown to have spoilage attributes, is capable of biofilm formation and can be resistant to preservatives such as sorbic acid, benzoic acid and sulphur dioxide (Las Heras-Vazquez *et al.*, 2003). *Lodderomyces elongisporus* is also very specifically frequently isolated a spoilage yeast in fruit juices being acid tolerant, xerophilic and extremely resistant to weak acid preservatives (Tournas *et al*., 2006).

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Indicated as band positions 14, 16, 31, 36, 38, 42 and 45 on the gel in Figure 4.14A, *L. elongisporus* was present in all six lanes, showing clear representation that both disinfectant B and E were incapable of inhibiting this organism or required more time to eliminate all cells present in the nozzles, and also that reversion to the old disinfectant it still remained. The interesting factor here is both disinfectants were able to inhibit this organism during the MIC test at relatively low concentrations of 0.025% for disinfectant B and 0.0125% for disinfectant E.

The importance of carrying out the facility trial in the true environment as opposed to lab scale testing is again demonstrated. One of the reasons that could offer an explanation as to why this yeast may be resistant/tolerant to these QAC disinfectants is co-cultures of yeasts may influence the protein load and mimic dirty conditions, affecting the efficacy of the disinfectants (Theraud *et al.*, 2004). Previous studies have shown that the "killing activity" of hypochlorite and QAC's on *Candida albicans,* another yeast from the same family capable of biofilm formation*,* was reduced in the presence of a high protein load. Much less is known about the efficacy of disinfectants in inactivating yeasts such as *Candida albicans, Cryptococcus* spp*.* and *Rhodotorula* spp. that contribute to biofilm formation in the environment.

Kazachstania exigua has been isolated from olive brine, kefir cultures (fermented milk drink), wine production, spoiled soft drinks, soil, strawberries and grape must. This yeast is often used in the production of sourdough (Pitt & Hocking, 2009). No biofilm formation capabilities were found to be reported however the organism has been reported as very preservative resistant, and capable of growth under very acidic conditions. It is also resistant to acetate, propionate and sorbate. This yeast has been found to have inhibitory effects against pathogenic bacterial species (Perez *et al.*, 2016).

Kazachstania exigua is represented by band positions 3, 5, 20, 23, 24 and 40 on the gel in Figure 4.10A, which was on numerous occasions present in A0-pooled, A1-pooled and A2-pooled, indicating this yeast was not able to be inhibited by disinfectant B, or it

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could also be possible that it would eventually be inhibited with prolonged exposure to the disinfectant as the last sampling event included reversion to the old disinfectant first. The yeast also showed presence in lane B2-pooled for the last sampling event of disinfectant E, again indicating reversion to the old disinfectant definitely illustrates no positive outcomes. Possible resistance/tolerance to disinfectant B could be related to the same reason why the yeast is very resistant to preservatives. Not much information currently describes how resistance is possible for *K. exigua.* Although, current information point to the fact that the yeast is highly acid tolerant explaining why it may be constantly present in the final product and therefore always present even after disinfection takes place (Pitt & Hocking, 2009). Certain answers would only be obtained if prolonged uninterrupted exposure to disinfectant B takes place. One of the reasons that could offer an explanation as to why this yeast may be resistant to these QAC disinfectants is co-cultures of yeasts may influence the protein load and mimic dirty conditions, affecting the efficacy of the disinfectants (Pitt & Hocking, 2009).

Pichia occidentalis with synonyms *Issatchenkia occidentalis* and *Candida sorbosa,* is most often associated with natural fermentation of food products (Kurtzman, 2003). It has been found to be relatively common in wine fermentations. Due to its association with fruit and other products it may also play a role in food spoilage. *Pichia occidentalis* has been found to possess a low to moderate ability to form biofilms (Sardi *et al*., 2013).

Indicated as band position 37 on the gel it is present in both of the last sampling events however, not the first. Again indicating no positive impact of reverting to the old disinfectant where inhibition had to once again commence. Though it must be noted that it could have been present in the first sampling events, but was just not part of the sampled pool due to every second nozzle being swabbed.

Rhodotorula dairenensis has been isolated from soil, plant material, water, milk, fruit juice and air. It has been found to be capable of biofilm formation disinfectant (Gadango & Sampaio, 2001; Nunes *et al*., 2012; Worth & Goldani, 2012).

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Displayed as band positions 29 and 33, this yeast was present in lanes A0-pooled and A1-pooled, and no longer in lane A3-pooled, showing disinfectant B was capable of inhibiting this yeast completely. However, it still remained present in lanes of B-pooled, indicating disinfectant E was incapable of inhibiting this organism at any sampling event and still also remained after reversion to the old

The genus *Sporidiobolus* consists of eight known species *S. johnsonii*, *S. longiusculus*, *S. metaroseus*, *S. pararoseus*, *S. ruineniae*, *S. ruineniae var. ruineniae*, *S. salmonicolor* and *S. veronae*. Isolations of species in the genus have been found from air, leaf of raspberry, grains, terrestrial plants, water reservoirs, flowers, garden soil and rotten wood (Bross *et al.*, 1986; Libkind *et al.*, 2005). Characteristics include opportunistic pathogens, biocontrol agents in post-harvest of fruits and biofilm formation capabilities (Sampaio, 2011; Huang *et al.*, 2012).

Band position 28 represents *Sporidiobolus* sp. on the gel in Figure 4.14A. It was always present at all three sampling events in filler B and absent in filler A. It may have been present at the first sampling events for disinfectant B, however was inhibited and did not resurface. Disinfectant E however did not seem to be capable of inhibiting this yeast, perhaps further prolonged exposure would result in inhibition as the band intensity did show a decrease with every sampling event.

Rhynchogastrema noutii has been isolated from fruit (Laqout) trees and dried unripe fruit. It has the well-known and more common synonym of *Bandoniozyma noutii* (Valente *et al.*, 2012)*.* This yeast was only detected in the nozzles of filler A during the last sampling event (A2-pooled) at band positions 19 and 35. It seemed to have appeared in filler A after the switch back to disinfectant A. It is difficult to say from where *R. noutii* was introduced into the system during the disinfectant changeover.

An uncultured fungus clone was also noted from the sequence analysis to be present in lanes B1-pooled and B2-pooled and although no longer there at lane B3-pooled for disinfectant E, indicated a band at band position 30 on the gel. It was not present in the

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samples from filler A. Further studies would be required to confirm the identity and characteristics of this organism. For the purpose of the study it did not show much cause for concern as even the perceived less effective of the two disinfectants seemed to be capable of inhibiting it.

4.4 Conclusion

Results of the facility disinfectant trial indicated three important factors. Firstly, the current disinfectant (disinfectant A) was not performing the function of inhibiting yeast survival, which were present at critical areas of direct food contact. Secondly, there is a possibility that the current disinfectant was so ineffective that it allowed for the formation of biofilms and development of resistance. Disinfectants B and E were able to disrupt and thereby inhibit problematic yeast as seen with the microbial growth results and heat maps analysis where considerable increases took place due to free cells present after biofilm disruption and then considerable decreases where the yeast were being inhibited. Thirdly, the use of an alternative medium for isolation and enumeration showed that the current facility process of microbial monitoring has shortfalls as the usual data trending rarely shows any cause for concern, due to the media currently used being inadequate at presenting the high levels of yeasts which are actually present in the nozzles. It is definitely suggested that the facility changes both the broth and agar currently in use and rather follow suite to the experimental YPD media which was used.

The facility produces products unaware of the possible dangers. When the opportunity arises the yeast which could be present in the environment and equipment or from raw materials, grow and cause spoilage, forcing the facility in certain cases to recall production batches. Investigation on possible causes does not show the condition of equipment or the vast presence of yeasts from the facility's microbial monitoring process. The facility then has to seek assistance from external sources to attempt to solve the issues. This can very easily change if the correct disinfectant is used for the disinfection

process as well as correct microbial monitoring media including further identification of any growth colonies obtained from equipment and or products takes place.

Disinfectant B has shown the ability to inhibit yeast growth in both lab scale and full scale testing. The disinfectant is readily available, effective, does not have any major influences on the process, does not require additional resources such as water or residue test kits than what the facility currently makes use of. In fact, disinfectant B was purchased from the same supplier as the current disinfectant in use.

Interestingly though disinfectant B outperformed disinfectant E in the facility trial process which is opposing to what was expected from the MIC test results where disinfectant E showed inhibition to the same 20 isolates tested at much lower concentrations than B. The possible explanation for this is disinfectant E's suggested contact time according to the manufacturer was 30 minutes, and due to this disinfectant being able to inhibit the 20 yeast isolates in the MIC test in 5 minutes, the same contact time was still applied. However, the disinfectant was unable to replicate its effect in the true up scaled environment where multiple yeasts are required to be inhibited simultaneously. Other factors which could have impacted performance of this disinfectant included nozzle design differences as well as type of products packed on the line as compared to filler A which are more favourable to yeast presence.

The molecular analysis results indicate that disinfectant B outperformed E in being able to inhibit yeast growth. The process did have an interruption (changed to current disinfectant for a month) which further presented interesting results as time three in the heat maps data. The only two yeasts which were of concern as not being inhibited by both disinfectants was *L. elongisporus* and *K. exigua*. It is likely, based on the MIC results for *L. elongisporus* and the mode of how resistance occurs, with co cultures that the uninterrupted use of disinfectant B would result in the inhibition of all yeasts present in equipment and nozzles (Theraud *et al*., 2004).

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With a change from the current disinfectant to disinfectant B on both fillers and the SOP for microbial monitoring, the facility will have the ability to truly determine problematic yeasts and act accordingly to prevent spoilage of beverages and maintain a hygienic facility and process equipment as opposed to reacting in times of crisis after recalls and or production/margin losses take place.

4.5 References

Araujo, P.A., Lemos, M., Mergulhao, F., Melo, F. & Simoes, M. (2013). The influence of interfacing substances on the antimicrobial activity of selected quaternary ammonium compounds. *International journal of food science*, Article ID 237581, 9 pages.

Bartlett, J.M.S. & Stirling, D. (2003). A short history of the polymerase chain reaction. *PCR protocols. methods in molecular biology* **226,** 3-6.

Borelli, G., José, J.P., José, Teixeira, P.L., dos Santos, L.V., Amarante, G. & Pereira, G. (2016). De Novo assembly of *Candida sojae* and *Candida boidinii* genomes, unexplored xylose-consuming yeasts with potential for renewable biochemical production. *American society for microbiology. Genomes announcements* **4**, E01551-15.

Bridier, A., Brainet, R., Thomas, V. & Deboius-Brissonnet, F. (2011). Resistance of bacterial bioflims to disinfectants: a review. *Biofouling* **27**, 1017-1032.

Bridier, A., Vizueta., S.P., Guilbard, M., Piard, J.C., Nataili, M. & Brainet, R. (2015). Biofilm associated resistance to food borne pathogens. *Food microbiology* **45**, 167-178.

Brockson, M.E., Novotny, L.A., Mokrzan, E.M., Malhotra, S., Jurcisek, J.A., Akbar, R., Devaraj, A., Goodman, S.D. & Bakaletz, L.O. (2014). Evaluation of the kinetics and mechanism of action of anti-integration host factor-mediated disruption of bacterial biofilms. *Molecular microbiology* **4***, 625-637.*

Bross, J.E., Manning, P., Kacian, D. & Talbot, G.H. (1986). Pseudomeningitis caused by *Sporobolomyces salmonicolor*. *American journal of infection control* **14,** 220–223.

Cocolin, L., Aggioa, D., Manzanoa, M., Cantonib, C. & Comi, G. (2001). An application of PCR-DGGE analysis to profile the yeast populations in raw milk. *International dairy journal* **12**, 407-411.

Dahm, R. (2008). Discovering DNA: Friedrich Miescher and the early years of nucleic acid research. *Human genetics* **122,** 565–581.

de Smidt, O., Smit, N.J. & Botes, E. (2014). Bacterial diversity in soil from geophagic mining sites in the Qwa-Qwa region of South Africa. *International journal of environmental health research* **25,** 184-195.

Deak, T. (1991). Foodborne yeast. *Advanced applied microbiology* **36,** 123-127.

Donlan, R.M. (2002). Biofilms, microbiological life on surfaces. *Emerging infectious diseases* **8**, 881- 890.

Dusane, D.H., Dam, S., Nancharaiah, S.V., Kumar, S.R., Venugopalan, V.P. & Zinjarde, S.J. (2012). Disruption of *Yarrowia lipolytica* biofilms by rhamnolipid biosurfactant. *Aquatic biosystems* **8**, 7 pages.

Fraser, F. (2003). Cleaning and sanitation. *Department of family and consumer sciences*, NC State University, Raleigh, NC 27695-7605.

Gadango, M. & Sampaio, P.J. (2001). Polyphasic taxonomy of the basidiomycetous yeast genus *Rhodotorula: Rh. glutinis* sensu stricto and *Rh. dairenensis* comb. nov. *FEMS yeast research* **2,** 47-58.

Havelaar, A.H. (1994). Application of HACCP to drinking water supply. *Food control* **5**, 145–152.

Hernández, A.F., Pérez-Nevadoa, S., Ruiz-Moyanoa, M.J., Serradillab, M.C. Villalobosa, A.M. & Córdobaa, M.G. (2018). Spoilage yeasts: What are the sources of contamination of foods and beverages? *International journal of food microbiology* **286**, 90-110.

Huang, R., Che, H.J., Zang, J., Yang, L., Jiang, D.H. & Li, G.Q. (2012). Evaluation of *Sporidiobolus pararoseus* strain YCXT3 as biocontrol agent of *Botrytis cinerea* on post-harvest strawberry fruits. *Biological control* **62**, 53-63.

Jang, H., Rusconi, R. & Stocker, R. (2017). Biofilm disruption by an air bubble reveals heterogeneous age-dependent detachment patterns dictated by initial extracellular matrix distribution. *Npj biofilms and microbioms* **3**, 502-513.

Julien, M.C., Dion, P., Lafreniere, C., Antoun, H. & Drouin, P. (2008). Sources of clostridia in raw milk on farms. *Applied environmental microbiology 74,* 6348–6357.

Kurtzman, C.P. (2003). Phylogenetic circumscription of *Saccharomyces*, *Kluyveromyces* and other members of the *Saccharomycetaceae*, and the proposal of the new genera *Lachancea, Nakaseomyces, Naumovia, Vanderwaltozyma* and *Zygotorulaspora. FEMS yeast research* **4,** 233-245.

Las Heras-Vazquez, F.J., Mingorance-Cazorla, L., Clemente-Jimenez, J.M. & Rodriguez-Vico, F. (2003). Identification of yeast species from orange fruit and juice by RFLP and sequence analysis of the 5.8S rRNA gene and the two internal transcribed spacers. *FEMS yeast research* **3,** 3– 9.

Libkind, D., Gadanho, M., van Broock, M. & Sampaio, J.P. (2005). *Sporidiobolus longiusculus* sp. nov. and *Sporobolomyces patagonicus* sp. nov., novel yeasts of the *Sporidiobolales* isolated from aquatic environments in Patagonia, Argentina. *International journal of systematic and evolutionary microbiology* **55,** 503-509.

Manning, L. & Baines, R.N. (2004). Effective management of food safety and quality. *British food journal* **106**, 598-606.

Marzorati, M., Wittebolle, L., Boon, N., Daffonchio, D. & Verstraete, W. (2008). How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environmental microbiology* **3**, 11 pages.

Mazzola, P.G., Jozala, A.F., Novaes, L.C., Moriel, P. & Vessoni- Penna, T.C. (2009). Minimal inhibitory concentration (MIC) determination of disinfectant and /or sterilizing agents. *Brazilian journal of pharmaceutical sciences* **45**, 241-248.

Mlimbila, J.S., Hosea, K.M. & Muruke, M. (2013). Molecular identification and proteinase activity of yeasts isolated from fermented milk*. International journal of dairy science research* **2**, 7-14.

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied environmental microbiology* **59,** 695-700.

Nakase, T., Suzuki, M., Takashima, M., Miyakawa, Y., Kagaya, K., Fukazawa, Y. & Komazata, K. (1994). *Candida sojae*, a new species of yeast isolated from the extraction process of water-soluble substances of defatted soy beans. *Journal of general applied microbiology* **40,** 161-169.

Niamsuwan, S., Kittisupakorn, P. & Mujtaba, I.M. (2011). Minimization of water and chemical usage in the cleaning in place process of a milk pasteurization plant. *Songklanakarin Journal of science and technology* **33**, 431-440.

Nunes, J.M., Bizerra, F.C., Ferriera, R.C. & Colombo, A.L. (2012). Molecular identification, antifungal susceptibility profile, and biofilm formation of clinical and environmental *Rhodotorula* species isolates. *Antimicrobial agents and chemotherapy* **57,** 382-389.

Olsvik, O., Wahlberg, J., Petterson, B., Uhlén, M., Popovic, T., Wachsmuth, I.K. & Fields, P.I. (1992). Use of automated sequencing of polymerase chain reaction generated amplicons to identify three types of Cholera Toxin subunit B in *Vibrio cholerae* 01 Strains. *Journal of clinical microbiology* **31**, 22-25.

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Pavlov, A.R., Pavlova, N.V., Kozyavkin, S.A. & Slesarev, A.I. (2004). Recent developments in the optimization of thermostable DNA polymerases for efficient applications. *Trends in biotechnology* **22,** 253-260.

Perez, M.F., Contreras, L., Garnica, N.M., Fernandez-Zenoff, M.V., Farıas, M.E. & Sepulveda, M. (2016). Native killer yeasts as bio control agents of postharvest fungal diseases in lemons. *Plos one* 11 **10**, E0165590. 21 pages.

Peters, S. (2015). 5 tips for getting the most out of your CIP system. *CIP systems, Crane engineering,* https://blog.craneengineering.net/5-tips-for-getting-the-most-out-of-your-cip-system.

Pettersson, E., Lundeberg, J. & Ahmadian, A. (2009). Generations of sequencing technologies. *Genomics* **93**, 105-111.

Pitt, J.I. & Hocking, A.D. (2009). *Fungi and food Spoilage*, 3rd edn. Springer Dordrecht Heidelberg, London: New York.

Salo, S. & Wirtanen, G. (2005). Disinfectants efficacy on foodborne spoilage yeast strains. *Food and bio products processing* **83**, 288-296.

Sampaio, J.P. (2011). *The Yeasts* 5th edn,. Elsevier Science, London.

Sardi, J.C.O., Scorzoni, L., Bernardi, T., Fusco-Almeida, M. & Mendes Giannini, M.J.S (2013). Candida species: current epidemiology, pathogenicity, biofilm formation, natural antifungal products and new therapeutic options. *Journal of medial microbiology* **62**, 10-24.

Scorzetti, G., Fell, J.W., Fonseca, A. & Statzell-Tallman, A. (2002). Systematics of basidiomycetous yeasts: a comparison of large subunit D1/D2 and internal transcribed spacer rDNA regions. *FEMS Yeast Research* **2,** 495–517.

Sharkey, D.J., Scalice, E.R., Christy, K.G., Atwood, S.M. & Daiss, J.L. (1994). Antibodies as thermolabile switches: High temperature triggering for the polymerase chain reaction. *Biotechnology* **12**, 506-509.

Tamime, A.Y. (2009). Cleaning-in-Place: Dairy, food and beverage operations, 3rd edn Society of dairy technology, Huntingdon, UK.

Theraud, M., Bedouin, Y., Guigen, C. & Gangneux, J.P. (2004). Efficacy of antiseptics on clinical and environmental yeast isolates in planktonic and biofilm conditions. *Journal of medical microbiology* **53**, 1013-1018.

Thomas, A. & Sathian, C.T. (2014). Cleaning-In-Place (CIP) System in dairy plant review. *Journal of environmental science, toxicology and food technology* **8**, 41-44.

Tournas, V.H., Heeres, J. & Burgess, L. (2006). Moulds and yeasts in fruit salads and fruit juices. *Food microbiology* **23**, 684–688.

United Kingdom. SPX Corporation. (2013) CIP and sanitation of process plant. Denmark: *SPX*, Print.

Valente, P.,Boekhout, T., Fontes Landell, M., Crestani, J., Pagnocca, F.C., Sette, L.D., Passarini, M.R.Z., Rosa., C.A., Brandao, L.R., Pimenta., R.S., Ribeiro, J.C., Garcia, K.C., Lee, C.F., Suh, S.O., Peter, G., Dlauchy, D., Fell, J.W., Scorzetti, G., Theelen, B. & Vainstein, M.H. (2012). *Bandoniozyma* gen. nov., a genus of fermentative and non-fermentative Tremellaceous yeast species. *PLOS one* **7,** Article ID e46060, 11 pages.

Worth, F. & Goldani, L.Z. (2012). Epidemiology of *Rhodotorula*: An emerging pathogen. *Interdisciplinary perspectives on infectious disease***,** Article ID 465717, 7 pages.

Chapter 5

Concluding remarks

Yeasts has the ability to cause spoilage in the food and beverage industry due to their ability to persist in the products as the products possess intrinsic characteristics favourable to the growth of these organisms. The beverage industry for concentrated beverages show this weakness where yeast are present and cause spoilage resulting in recalls, product losses, profit decreases and drops in consumer confidence in associated brands.

The need for this study arose when a processing facility producing concentrated beverages raised a concern about reoccurring spoilage that results in thousands of litres being recalled. A previous study investigated the yeast diversity present in the environment and equipment of the processing facility and isolated yeasts where the most concerning occurrence included equipment after disinfection took place as well as in final products. The presence of yeast after CIP alluded to the fact that inadequate disinfection was in all probability linked to the disinfectant in use.

The disinfectant that was in use at the facility as well as other readily available disinfectants with different active ingredients and modes of actions were tested against 20 yeast species isolated from the equipment and fruit juice. The 96 well MIC testing protocol successfully identified two disinfectants that showed outstanding performance in being able to inhibit all 20 yeast isolates. From the two identified disinfectants B and E, disinfectant E in particular, showed much potential not only because it was capable of inhibition of all 20 yeast isolates at very low concentrations, but also because its use implied a considerable cost reduction towards disinfection for the facility. To implement disinfection at a reduced cost would of course always be received in the food industry as profit margins are often very narrow. The concern noted, however, was the high foaming characteristic of disinfectants B and E that could possibly lead to an unwanted increase in the volume of rinse water during the disinfection process.

Disinfectants B and E ($DDAC_2$) used in a facility trial process on two different production lines and monitored over a period of seven months. Microbial monitoring was performed

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in the filler nozzles as they were identified as the niches most likely able to promote and harbour biofilms. Heat maps of the changes in yeast numbers clearly displayed the efficacy of the trial disinfectants in lowering the yeast load in the filler nozzles. The initial yeast counts did show increases which was due to the destruction in biofilms taking place releasing free cells and therefore increasing counts. Later on, the counts showed great reduction and most interestingly the results were opposing to which was anticipated from the MIC test results. This facility trial also identified a shortcoming in the microbial monitoring protocol currently being used in the facility. The liquid media and culture agar used resulted in an under estimation of the actual yeast load which in turn did not indicate any concerns based on results obtained during regular monitoring and trending. Therefore, only reactive measures could take place when spoilage of final products was reported.

Disinfectant B performed best during the facility trial despite the fact that it contained a lower concentration of $DDAC_2$. It was able to reduce yeast counts in the nozzles to almost undetectable levels within 3 months (only 2 nozzles retained growth). The colonies obtained from the microbial monitoring process were also subjected to molecular analysis to investigate the diversity changes in the yeast population during the trial process. The molecular analysis demonstrated limited diversity with only eight species detected; *Candida sojae*, *Lodderomyces elongisporus*, *Kazachstania exigua, Pichia occidentalis, Rhodotorula dairenensis, Sporidiobolus sp., Rhynchogastrema noutii* and an uncultured fungus clone. Two species in particular raised concern as neither disinfectant B nor E was able to eliminate them completely. These were *L. elongisporus* and *K. exigua* possibly tolerant or resistant to $DDAC_2$ to when occurring in complex communities for further prolonged exposure required due to resistance/tolerance measures.

The study therefore undoubtedly proved the initial hypothesis that the disinfection process was inadequate in using an unsuitable disinfectant which lead to high yeast loads and biofilm development in the filler nozzles. The study also successfully identified

a replacement disinfectant with the potential to eliminate yeast which develop biofilms and lead to spoilage of products.

Various aspects were also highlighted that would be worth investigating in future. These include:

- Making use of disinfectant B in the testing facility over prolonged uninterrupted periods to determine if any other resistance is present or can develop.
- Performing MIC testing on the newly identified yeasts found during the facility trial
- A time trial to monitor the development of resistance to disinfectants and how this can be prevented.
- Further study the uncultured fungal clone obtained during the facility trial to determine if a new species has been detected.