Diversity profiling and rapid detection of spoilage yeasts in a typical fruit juice bottling factory

By

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Declaration of independent work

I, Kereng Merona Corbett (student number [redacted]), hereby declare that this research project submitted to the Central University of Technology, Free State, for the degree Master of Health Science in Environmental Health is my own independent work. This research project was conducted at the Central University of Technology, Free State under the supervision of Dr O de Smidt and co-supervised by Prof. JFR Lues.

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Dr Olga de Smidt

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Abstract

Spoilage caused by yeasts is a constant and widespread problem in the beverage industry which can result in major economic losses. Fruit juices provide an environment which allows the proliferation of yeasts, leading to spoilage of the product. Some factories do not have the laboratory facilities to identify spoiler yeasts and it becomes a prolonged process if outsourced, which obstructs the planning of corrective actions. This study aimed to establish yeast diversity and apply a rapid method for preliminary identification of spoiler yeasts associated with a small scale fruit juice bottling factory. The yeast population in the factory was determined by isolating yeasts from the production environment, process equipment and the spoiled products. Yeasts were identified by PCR-RFLP analysis targeting the 5.8S-ITS region and sequencing the D1/D2 domain of the 26S rRNA gene. A total of 201 yeasts belonging to ten different genera (*Candida, Lodderomyces, Wickerhamomyces, Yarrowia, Zygosaccharomyces, Zygoascus, Cryptococcus, Filobasidium, Rhodotorula/Cystobasidium* and *Trichosporon*) were isolated and identified from the production environment and processing equipment. The overall yeast distribution showed that *Candida parapsilosis* and *Lodderomyces elongisporus* were widely distributed in the factory, with *Candida parapsilosis* being reported as an opportunistic pathogen. *Zygosaccharomyces bailii*, *Zygoascus hellenicus* and *Saccharomyces cerevisiae* were isolated from the spoiled products and are known to be highly fermentative. In addition, *Zygosaccharomyces bailii* and *Zygoascus hellenicus* were found to be present inside the refrigerator where the fruit pulp is stored, which makes it a potential point of contamination. The data also provided a yeast control panel which was successfully utilized to identify unknown yeast in spoiled product from this factory using PCR-RFLP analysis.

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List of abbreviations

%	Percentage
ATP	Adenosine Triphosphate
a_w	Water activity
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
CFU	Colony-forming unit
CFU/ml	Colony-forming units per millilitre
CIP	Clean-in-place
cm^2	Square centimetre
EDTA	Ethylene diamine tetra acetic acid
FDA	Food and Drug Association
FT-IR	Fourier transform infrared spectroscopy
gDNA	genomic DNA
GFSI	Global Food Safety Initiative
GMP	Good Manufacturing Practices
GRAS	Generally Regarded As Safe
h	Hour
HACCP	Hazard Analysis and Critical Control Points
HCL	Hydrochloric acid
HPP	High Pressure Processing
HTST	High Temperature/ Short Time
IGS	Non-transcribed Intergenic Spacers
ITS	Internal Transcribed Spacer

ISO	International Organization for Standardization
Kb	Kilobase
LTLT	Low Temperature/ Long Time
MALDI	Matrix-assisted laser desorption/ionization
MALDI-TOF MS	Matrix Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry
MEA	Malt-Extract Agar
mg/kg	Milligrams/Kilograms
min	Minutes
ml	Millilitres
mm	Millimetres
mM	Millimolar
MPa	Megapascal
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
nm	Nanometers
ºC	Degree Celsius
PCR	Polymerase Chain Reaction
PMA	Phorbol 12-myristate 13-acetate
ppm	Parts per million
RBC	Rose Bengal Chloramphenicol
RFLP	Restriction Fragment Length Polymorphism
RH	Relative humidity
RNA	Ribonucleic acid
rpm	Revolutions per minute

rDNA	Ribosomal DNA
rRNA	Ribosomal RNA
SABS	South African Bureau of Standards
sec	Seconds
U	Unit
US	Ultrasound
UV	Ultraviolet
v/v	Volume/volume
w/w	Mass/mass
YM	Yeast – extract malt glucose
µl	Microlitre
µM	Micrometre

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Chapter 1

Introduction

1.1 Background

Fruit juices are important commodities in the global food market providing vast possibilities for new value added products to meet consumer demand for convenience, nutrition and health (Aneja et al., 2014). The food and beverage industry is one of the most important components of South Africa's manufacturing sector. Beverages account for just over 4% of all manufacturing sales and within the food and beverage sector, beverages accounts for 24% of sales (FoodBev–SSP update, 2013). Increased consumption of fruit juices has a direct influence on the economy in a positive way, but becomes negatively affected when foodborne disease outbreaks and spoilage problems occur (Tribst et al., 2009).

A major goal for any food processing industry is to provide safe, wholesome and acceptable food to the consumer. Control of microorganisms is essential to meeting this goal. Extensive measures have been taken to control the threat of yeast spoilage, especially in the fruit juice bottling industry. Growth of yeasts in fruit juices is governed largely by a series of physical and chemical parameters. Consequently, management of the environment of foods can change these factors and delay spoilage. Control over spoilage caused by yeasts is partly exerted through processing and preservation techniques that eliminate these microorganisms or prevent their growth (Bagge-Ravn, 2003; Suárez-Jacobo et al., 2010). However; recent demands by consumers for preservative-free and safe foods which have then undergone milder processing while maintaining their extended shelf-life impose new challenges to the food industry (Lucera et al., 2012).

Microbiota are also endlessly innovative and eventually seem to circumvent the barriers setup against them. Most food industries incur high costs in an attempt to prevent or reduce microbial spoilage. Quality control in bottling factories entails rigorous microbial

monitoring of pulps, water, air and equipment. Yet, yeast spoilage problems still occur. Moreover, the number of microorganisms capable of tolerating the environmental conditions present in the food processing, including the ability to resist and overcome preservative measures used to control spoilage, has increased dramatically.

In order to design adequate strategies to prevent spoilage, it is advantageous to know the identity of the spoilage organisms present in the product and to get an insight into the source of contamination (Loureiro, 2000). Improved techniques with increased specificity, discriminatory power and shorter detection times for the identification of spoilage yeasts in foods and drinks are becoming increasingly important in the food sector (Casey and Dobson, 2004). Knowing the identity of the yeast causing spoilage, as well as its location in the factory will allow for the quicker implementation of intervening measures, thus reducing the effects of potential spoilage while also providing a means of monitoring quality control in production processes.

1.2 Aim and objectives

A prominent fruit juice bottling factory in Bloemfontein experiences an annual problem with ‘blowing’ of the fruit juice concentrates. Furthermore, the establishment experienced difficulty of long waiting periods for identification of the spoilage contaminants which delayed corrective actions. Therefore, the aim of this research was to compile a yeast diversity profile of this fruit juice bottling factory and to apply a rapid method for preliminary identification of spoilage yeasts associated with its products. In order to meet the aim the following objectives were devised:

- Establish yeast diversity by isolating yeasts from different areas in the factory
- Identify yeast isolates by PCR and 26S rRNA D1/D2 domain sequencing
- Perform 5.8S rRNA region RFLP analysis as a possible method for identifying spoilage yeasts from ‘blowing’ products

1.3 Project layout

Chapter 1: Introduction

Chapter 2: Literature review highlighting fruit juice spoilage yeasts, their implications and control.

Chapter 3: Discusses the different yeasts isolated and identified in the factory, as well as distribution.

Chapter 4: Evaluation of RFLP as a possible preliminary identification method for spoilage yeasts detected in fruit juice.

Chapter 5: Summative remarks, conclusions, recommendations and future research

Chapter 2
Literature review

2.1 Introduction

Food production has become more complex during the past decade; production volumes are larger, operations are more mechanical, food is more processed and the time and distance between production and consumption longer (Lowes et al., 2000). Food borne disease and microbial spoilage of food result from failure or inability to control microorganisms at one or more stages of the food chain; from raw material production to consumption of the final product (Jaeyola et al., 2011). The implications of situations that result in food poisoning outbreaks or food spoilage can be severe for food producers, retailers, consumers and regulatory authorities (Arias et al., 2002). Potential sources of contamination of spoilage microorganisms are from water, air, insects and dirty contaminated equipment within food processing factories (Fleet, 1990; Deak and Beuchat, 1996; Tournas, 2006). This poses a challenge for the food industry since the quality and safety of products is negatively impacted and thus, rejected by consumers. Food products are usually produced in large quantities and this results in a major economic loss when the products are affected by spoilage (Loureiro, 2000).

It is estimated that one-third of the food world production is lost by microbial activity, which is an economically significant problem for manufacturers, retailers and consumers (Lund et al., 2000). Because of the particular environmental conditions in a food system, only a small proportion of microorganisms present will be able to grow rapidly and cause spoilage. Juice concentrates are more stable than other juice products; high sugar concentrations, low water activity and low pH preserve these products (Combina et al., 2008). However, the combination of these factors supports the development of a reduced number of microorganisms such as osmophilic or xerotolerant yeasts. It may be assumed that each type of food may be altered by a specific group of yeasts (Loureiro, 2000). These selected species could thus be considered as potentially harmful in this

context, and should be specifically controlled in the particular food industry. The rapid identification of spoilage yeasts is of great importance to the food industry. Based on the classical taxonomy criteria, a simplified identification key for yeast species associated with foods has been proposed by Deak (1986, 1992). On the other hand, several molecular-based methodologies have also been proposed to identify these yeasts (Loureiro and Querol 1999; Kurtzman, 2015). Their economic importance as spoilage organisms makes rapid identification high priority because only when the nature of the spoilage is understood can an informed decision be made on cleaning procedures and product recall (James and Stratford, 2003; Rawsthorne and Phister, 2006; Harrison et al., 2011). Identification is normally done by sequencing the ITS or D1/D2 rDNA regions (Hulin and Wheals, 2014). However, although this is a rapid procedure it is relatively expensive for large scale work and unless sequencing facilities are on site, it may take some days to get the results.

2.2 Fruit juice

Fruit juice is mainly composed of water (Sun, 2009). Another common constituent is carbohydrates which comprises of sucrose, fructose and glucose (Llamas et al., 2011). Additionally, the amount of proteins and minerals is limited in fruit juice and juice contains no fat or cholesterol. Apart from sugars, vitamins and minerals, fruit juices contain dietary fibre (Kregiel, 2015). The quality of fruit juices is highly affected by the organic acid profile. Major organic acids which compose of the 90% or more of the total acidity in grapes are tartaric and malic acids (Soyer et al., 2003). Citric and malic acids were reported to be the main acids in citrus fruits (Karadeniz, 2004). Ascorbic acid (Vitamin C) is usually present in a large variety of commercial fruit juices (Llamas et al., 2011). Citrus fruits and juices are good sources of ascorbic, folic acid, vitamin B1, thiamine and potassium (Zhang et al., 2011). A cup of citrus juice (240 ml) provides vitamin C in the quantity of more than daily requirements (Bates et al., 2001).

According to the Codex General Standards for fruit juices and nectars, fruit juice is the unfermented but fermentable liquid obtained from the edible part of sound, appropriately

mature and fresh fruit or fruit maintained in sound condition by suitable means including post-harvest surface treatments applied in accordance with the applicable provisions of the Codex Alimentarius Commission. Some juices may be processed with pips, seeds and peels which are not usually incorporated in the juice, but some parts or components which cannot be removed by Good Manufacturing Practices (GMP) will be acceptable (CODEX STAN 247-2005; FAO/WHO Food Standards, 2005).

The juice is prepared by standardized processes, which maintain the essential physical, chemical, organoleptic and nutritional characteristics of the juices of the fruit from which it comes (CODEX STAN 247-2005; FAO/WHO Food Standards, 2005). The juice may be cloudy or clear and may have restored aromatic substances and volatile flavour components, all of which must be obtained by suitable physical means, and all of which must be recovered from the same kind of fruit (R 1111 of 2005: Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972). Pulp and cells obtained by suitable physical means from the same kind of fruit may be added. A single juice is obtained from one kind of fruit. A mixed juice is obtained by blending two or more juices or juices and purées from different types of fruit.

2.3 Yeast spoilage

Food spoilage is frequently the result of microbial activity and the microorganisms that will proliferate in the product highly depend on the microbiota that come into contact with the ingredients and the product. Environmental conditions such as the composition of the product and the storage conditions also play a role in the occurrence of spoilage (Loureiro and Malfeito-Ferreira, 1993; Argyri, 2014). Yeasts are ubiquitous microorganisms that are often associated with the spoilage of a large variety of products in the food and beverage industries (Makino et al., 2010). Since yeasts can generally resist extreme conditions better than bacteria, they are often found in low pH products and those products containing preservatives to such an extent that bacteria cannot grow (Deak and Beuchat, 1996). Yeast spoilage in particular has increased in recent years as

a result of lower doses of preservatives and milder preservation processes which are required for higher standards of food quality (Koç et al., 2007).

Fruit juices represent an important market within the food industry and the increasing variety of products being released at a baffling rate has altered the potential for spoilage problems (Wareing and Davenport, 2008). These products contain key nutrients which encourage proliferation of spoilage yeasts (Stratford, 2006). On industrial scale, sugar syrups or syrups of fruit juices are frequently spoiled by osmotolerant yeasts which occur undetected as surface films on syrups stored in metal tanks (Sperber and Doyle, 2010). This may be governed by changes in temperature causing condensation on the metal above the headspace, diluting the surface layer and aiding faster yeast growth. This type of spoilage is difficult to detect in the bulk of the syrup and only emerges as a heavily contaminated layer when the tank is drained. As most beverage containers are only 300 – 400 mm height, the majority of yeast cells will have formed sediment within a few days and subsequent yeast growth forms thick sediment or a few visible colonies if the inoculum is small (Stratford, 1992). Yeast sediment may be easily visible to the consumer, but if the total volume of yeast is dispersed through the beverage it is largely undetected.

The most visible sign of yeast spoilage is the production of excess gas, leading to bulging containers or Tetra Paks, ‘blown cans’ or in extreme cases, exploding glass bottles, which can result in physical injury (Grinbaum et al., 1994; Martorell et al., 2007). Swelling of the product container due to gas production is a result of fermentation of sugars and is termed ‘blowing’. Furthermore, the volume of gas produced, and hence the pressure formed, varies depending on the yeast species producing it. The few yeast species capable of forming sufficient gas pressure by fermentation to explode bottles include *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae*, *Dekkera bruxellensis* and *Saccharomycodes ludwigii* (Stratford, 2006).

2.4 Yeasts as spoilage microorganisms in fruit juice

Products that have high sugar contents are targets for spoilage by yeasts (Ridawati et al., 2010). The medium of these products is usually linked with low pH, low oxygen levels and a high sugar concentration which prevent the growth of most microorganisms. However these hurdles do not inhibit the growth of osmophilic yeasts. These yeasts are described as being osmophilic or osmotolerant because they are able to survive in a habitat restricted to a high solute (e.g. sugar) environment (Stratford, 2006).

High sugar foods include honey, jams, sugar syrups, fruit juices and crystallized fruits (Rojo et al., 2015). They contain more than 67% sugar (w/w) and are prone to spoilage by osmophilic yeasts together with some xerotolerant species (Tilbury, 1980; Kuang et al., 2015). Osmotolerant yeasts are able to grow at 50% (w/w) sugar and at a water activity (a_w) of 0.88 while osmophilic yeasts are capable of growth at 60% sugar (w/w). Xerotolerant or xerophilic yeasts are also capable of growing at a low a_w of 0.62.

The most common yeast contaminants isolated from fruit concentrates are *Candida* spp., *Debaryomyces hansenii*, *Hansenula* spp., *Rhodotorula* spp., *Pichia* spp., *Dekkera* spp., *Lodderomyces elongisporus*, *Hanseniaspora* spp., *Issatchenkia orientalis*, *Kloeckera* spp., *Kluyveromyces marxianus*, *Pichia anomala*, *Saccharomyces* spp., *Torulaspora delbrueckii* and *Zygosaccharomyces* spp. (Sancho et al., 2000; Maciel et al., 2013). *Saccharomyces cerevisiae* is also considered to be a predominant spoilage species in concentrates, juices and fruit beverages and in that respect is considered to be the source of most problems associated with processed fruits (Sancho et al., 2000).

Zygosaccharomyces is a genus associated with the most extreme spoilage yeasts (Steels et al., 2000; Rojo et al., 2015). These yeasts are osmotolerant, fructophiles (prefer fructose), highly fermentative and extremely preservative-resistant. They usually grow slowly, producing off-odours, flavours and carbon dioxide that may cause food containers to swell and burst (Rawat, 2015). *Zygosaccharomyces rouxii* is one of the most extreme osmophilic microorganisms known, causing spoilage in sugar syrups and concentrates (Rojo et al., 2015). *Zygosaccharomyces mellis* is similarly osmophilic and causes spoilage of honey. Furthermore, *Zygosaccharomyces bailii* and

Zygosaccharomyces bisporus are the principle cause for concern in preserved foods due to their phenomenal resistance to preservatives (Davenport, 1997; Stratford et al., 2000).

Unlike bacteria, viruses and some filamentous fungi, yeasts are rarely associated with outbreaks of foodborne gastroenteritis, other foodborne infections or intoxications (Kurtzman et al., 2011). As part of normal daily food consumption, humans are unknowingly and inadvertently ingesting large, viable populations of a diversity of yeast species without significant adverse impact on their health (Querol and Fleet, 2006). These include yeasts in many cheeses, fermented and cured meats, fruits and fruit salads, home-brewed beer and wine. Nevertheless, an open mind and vigilance on yeasts and foodborne disease is required.

Yeast presence in foods has been associated with the onset of a broad range of allergic and hypersensitive reactions in humans (Querol and Fleet, 2006). Yeasts are not known as aggressive infectious pathogens when compared to bacteria and viruses. However, although the majority of yeasts are not acknowledged to be pathogens, various species are considered as opportunistic pathogens (Makino et al., 2010). A growing population of immuno-suppressed patients has resulted in increasingly frequent diagnoses of invasive fungal infections, including those caused by unusual yeasts (Miceli et al., 2011). Moreover, increasing numbers of yeast species, other than *Candida albicans* and *Cryptococcus neoformans* have been associated with these infections and are now considered in the list of opportunistic pathogens. These include yeast species that are frequently found in foods such as *Candida krusei*, *Issatchenkia orientalis*, *Pichia anomala*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae,* *Trichosporon* and various *Rhodotorula,* species (Makino et al., 2010; Miceli et al., 2011). *Saccharomyces cerevisiae* is now considered to be an emerging opportunistic pathogen, which has been addressed on several occasions (De Llanos et al., 2011).

Although the consumption of food contaminated with yeasts may not have a direct role in causing opportunistic infections, there is also an increasing concern that food may be an underestimated source of environmental pathogens (Wirth and Goldani, 2012). It is also possible that foods could be a source of yeasts that colonize the intestinal tract,

from where they translocate to the blood system, resulting in fungaemia and distribution to infect various organs (Cole et al., 1996).

2.5 Sources of contamination

Microbial contamination may originate from any step along the beverage manufacturing process (Guillamón et al., 1998; Vasavada, 2010). Raw materials, factory environment, dirty packages and unhygienic process equipment are all potential contamination sources (Stratford, 2006). In order to produce microbiologically safe and stable beverages, controlling raw material quality is necessary. Post-harvest sources of fresh produce include harvesting equipment, human handling, rinse water, transport vehicles and processing equipment (Burnett and Beuchat, 2001; Vasavada, 2010). Chemical and physical treatments are usually used to ensure the quality of beverage and process water (Lawlor et al., 2009). If treated improperly, water may bring spoilage microbiota to the process areas and to the final product. Process waters, especially contaminated cooling and rinsing waters, are common sources of yeasts in beverages (Stratford, 2006).

Sweeteners and sugar have also been reported as sources of spoilage organisms (Davenport, 1996; Sperber and Doyle, 2010). Sweeteners used in the beverage industry are typically syrups. They contain on average 67 °Brix and have a low water activity. Mainly, osmophilic yeasts may grow in these syrups. Low water activity controls the growth of yeasts, and therefore it is important to prevent condensate formation in syrup storage tanks and containers (Juvonen et al., 2011). Drops of condensate water may establish microenvironments with higher water activity and lead to a rapid increase in yeast growth rate (Lawlor et al., 2009).

It has been estimated that poor factory hygiene accounts for 95% of beverage spoilage incidences caused by yeasts (Juvonen et al., 2011). Secondary contaminations may arise from the factory environment and dirty processing equipment such as packaging, filling and capping machines, conveyors, soap, lubrication systems, meters and proportioning pumps and valve seals (Stratford, 2006). Returnable glass bottles can also be a significant source of spoilage microbes (especially yeasts) in the factory

environment (Lawlor et al., 2009). Poor sanitary design, improper cleaning and sanitation procedures favour build–up of spoilage microbiota within the factory and increase the contamination and spoilage risk of final products (Stratford, 2006). Microorganisms can also attach easily onto the manufacturing surfaces (e.g. processing pipes, feeding lines), forming biofilms which are difficult to clean.

2.6 Factors affecting spoilage and shelf-life

2.6.1 Intrinsic factors

Intrinsic factors are the physical, chemical and structural properties inherent in the food itself. These are the properties of the final product (Bari, 2009). The most important intrinsic factors are water activity, pH, redox potential, available nutrients and natural antimicrobial substances (Huis in't Veld, 1996). The pH of the food and its ability to resist pH change (buffering capacity) together with mechanical barriers to microbial invasion are also part of this group (Garbutt, 1997). Intrinsic factors are influenced by variables such as raw material type and quality as well as product formulation and structure (Bari, 2009).

2.6.2 Extrinsic factors

Extrinsic factors are factors in the environment in which the food is stored, notably temperature, humidity and atmosphere composition (Huis in't Veld, 1996; Argyri, 2014). Time is also included because under any given set of circumstances, spoilage takes a finite period to occur and links with the storage life of a product (Garbutt, 1997). Factors which the final product encounters as it moves along the food chain also form part of extrinsic factors and are listed in Table 2.1.

Table 2.1 Extrinsic factors in the environment in which the food is stored and the stages at which they occur (Adapted from Bari, 2009)

Extrinsic factor	Stage
Time-temperature profile	Processing
Temperature control	Storage and distribution
Relative humidity (RH)	Processing, storage and distribution
Exposure to light (Ultraviolet and Infrared)	Processing, storage and distribution
Environmental microbiological counts	Processing, storage and distribution
Subsequent heat treatment	e.g. Reheating or cooking before consumption
Consumer handling	Household

2.6.3 Implicit factors

Implicit factors are those physiological properties that enable particular organisms to flourish resulting from the interaction of factors such as the intrinsic and extrinsic (Argyri, 2014). These factors are the result of the development of a microorganism which may have a synergistic or antagonistic effect on the microorganisms present in the food product (Mossel et al., 1995). Synergistic effects involve the production or availability of essential nutrients as a result of the growth of a certain group of microorganisms which allows the development of other microorganisms which were initially unable to grow (Huis in't Veld, 1996). Similarly, changes in pH value, redox potential and water activity may enable the development of microorganisms less tolerant to these inhibitory factors, yielding secondary spoilage.

Antagonistic processes include competition for essential nutrients, changes in pH value or redox potential, the formation of antimicrobial substances e.g. bacteriocins which may negatively affect the survival or growth of other microorganisms (Stiles and Hastings, 1991; Valero et al., 2012). An additional significant phenomenon in food preservation is the homeostasis of microorganisms (Gould, 1988; Shalini and Singh, 2014). If the

homeostasis of a microorganism i.e. their internal equilibrium is disturbed by preservative factors in foods, they will not multiply but they will remain in the lag phase or even die before their homeostasis is re-established.

2.7 Economic implications of spoilage

Most experiences of yeast spoilage of products are at the industrial scale. This problem is not unusual and the financial losses can be quite high (Arias et al., 2002; Rodrigues et al., 2012). Products such as fruit pulps and fruit juices are often implicated. When spoilage in foods is reported, the company involved may take numerous courses of action. Major incidents, particularly involving highly fermentative yeasts, may require a public recall of all products involved (Stratford, 2006). This may even extend to television advertising and requests to customers to return products to the shop. Such incidences are rare, but highly damaging to the brand image of companies concerned (Fleet, 2011). Minor incidents may be on a smaller scale, or may only involve spoilage to a lesser degree. In Europe, soft drink lines can run at 30,000 bottles per hour for approximately 16 h a day in summer and it has been noted that yeast spoilage is more prevalent in summer (Stratford, 2006). Recalling bottles from the infected line could run to more than ten million items.

For reasons of commercial confidentiality, the incidence and economic cost of industrial outbreaks of yeast spoilage remain unreported (Loureiro and Querol, 1999; Loureiro, 2000). In documenting the costs of such outbreaks; consideration needs to be given to the value of the spoiled product, the cost of recall and disposal, successive decreased retailer and consumer purchase of the product because of tarnished reputation and the legal and insurance fees associated with determining responsibility and awarding compensation (Fleet, 1992; Fleet, 2011). As a rule, several parties represented by manufacturers, suppliers of raw material and packaging and retailers are involved in these cases.

As per South African legislation, recalls by the food industry are initiated voluntarily in the interest of public safety. However, section 2(1) of the Foodstuffs, Cosmetics and

Disinfectants Act, No. 54 1972 (Act No. 54 of 1972) prevents any person from selling food that is unfit for human consumption. The regulations relating to the powers and duties of inspectors and analysts conducting inspections and analysis on foodstuffs and food premises (R 328 of 2007), as promulgated under the Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972, make provision for Environmental health inspectors to detain, sample and if necessary seize any foodstuff, in their areas of jurisdiction, which is deemed harmful or injurious to human health. National policy does not currently address official food product recalls. As a result, it has become challenging for food control authorities to execute, monitor and record any official food product recalls that may need to be conducted in South Africa (Policy guidelines, 2004).

2.8 Maintaining product quality and control measures

A number of methods of prevention can be used that can totally prevent, delay, or otherwise reduce food spoilage (Gould, 1996; Alzamora et al., 2011). Preservatives can expand the shelf life of food. In general, more than one method of preservation will be employed to achieve an acceptable shelf-life for products (Prokopov and Tanchev, 2007; Shalini and Singh, 2014). The use of different preservation methods is influenced not only by the shelf-life but also by the demand to preserve sensorial characteristics and nutritional value of the food, as some methods may significantly affect these aspects.

Contamination control measures include maintaining the cleanliness of equipment, the control of storage temperature, hot water immersion, chemical sanitizers, surfactants, surface waxes (particularly in oranges) and UV irradiation (Wareing and Davenport, 2008). Pasteurization and/or low-temperature storage protocols are also used to reduce the number of microorganisms in the final product (Arias et al., 2002). The chemical preservative dimethyldicarbonate (Velcorin) has been used to ‘cold pasteurize’ fresh juice products to reduce microbial loading, minimizing the use of sulphur dioxide (Juvonen et al., 2011). High–pressure processing, pulsed electric field and ultraviolet

radiation have also been investigated as novel control measures (Chen et al., 2013; Aneja et al., 2014).

Modeling of yeast and bacterial growth has been used as a technique for determining effective preservative and other control regimes. Good hygienic practices, adherence to Good Manufacturing Practices (GMP), implementation of food safety management systems and regulatory auditing have been shown to be effective control measures for microbial contamination in the beverage industry, particularly for yeasts (Wareing and Davenport, 2008; Duan, 2012). For example, the Hazard Analysis and Critical Control Point (HACCP) approach, ISO and GFSI have been adopted by food processors around the world as a food safety management system.

2.8.1 Inhibition of yeast growth by employing chemical preservatives

Many chemicals will eradicate microorganisms or stop their growth, but the majority of these are not permitted in foods (Saad et al., 2005). Chemicals that are permitted as food preservatives in South Africa are listed in Table 2.2. Chemical food preservatives are those substances which are added at very low quantities (up to 0.2%) and which do not alter the organoleptic and physico-chemical properties of the foods (Tfouni and Toledo, 2002). Preservation of food products containing chemical food preservatives such as sorbic and benzoic acid is usually based on the combined or synergistic activity of several additives, intrinsic product parameters and extrinsic factors (Jay, 2005). This approach minimizes undesirable changes in product properties and reduces concentration of additives and extent processing treatments.

Table 2.2 Chemical food preservatives (R 60 of 2009: Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972)

Foodstuff	Preservative	Quantity permitted
Fruit juices	Benzoic acid	600 mg/kg
	Propyl-p-hydroxy benzoate	1000 mg/kg
	Methyl-p-hydroxy benzoate	1000 mg/kg
	Sulphur dioxide	450 mg/kg
	Sorbic acid	600 mg/kg
	Pimaricin	5 mg/kg
Pineapple juice	Sulphur dioxide	10 mg/kg
	Pimaricin	5 mg/kg

2.8.1.1 Preservatives (Sorbic and benzoic acid)

Chemical preservatives such as sodium benzoate and potassium sorbate are often used to prevent microbial spoilage of fruit juices (Theron and Lues, 2010; Aneja et al., 2014). According to South African legislation, sorbic and benzoic acids are permitted food preservatives (R 60 of 2009: Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972). In other countries such as the USA, both acids have a generally recognized as safe (GRAS) status and can be found at higher concentrations. It is common and permitted to encounter mixtures of sorbic acid and benzoic acid in foods, both at reduced concentrations (Stratford, 2006). Membrane, respiration pathways and glycolysis metabolism are alternative sites of action for sorbic and benzoic acids. It has been suggested that sorbic acid is more inhibitory to yeasts than benzoic acid, conversely, benzoic acid is better than sorbic acid at pH 3. Sorbic acid is a six-carbon unsaturated

fatty acid unsaturated in positions 2 and 4, while benzoic acid comprises a carboxylic acid substituted into a benzene ring (Steels et al., 2000). These acids have little taste when used and a proven record of safe human consumption and lack of genotoxicity (Ferrand et al., 2000).

Since the free acids of both sorbic and benzoic acids are difficult to dissolve, these preservatives are normally added to foods as soluble salts, sodium benzoate and potassium sorbate, respectively. Both sorbic and benzoic acid are weak acids and inhibit microbes only in the undissociated form which prevails at low pH levels (Hazan et al., 2004). Undissociated, lipid-soluble acid molecules are able to diffuse rapidly through the microbial plasma membrane and into the cytoplasm. Within the cytoplasm weak acid molecules dissociate causing the cytoplasmic pH to decrease (Steels et al., 2000; Stratford, 2006). This decrease in cytoplasmic pH has been demonstrated for acetic acid and sulphite. However, sorbic acid has been calculated not to release sufficient protons to act as a weak acid preservative (Stratford and Anslow, 1998). The effect of sub inhibitory concentrations of preservatives is to cause slower growth of yeasts, smaller size, and much reduced cell yields.

2.8.1.2 Resistance of yeasts to preservatives

Preservative-resistant yeasts are a major concern for fruit juice and beverage industries. The main species playing a role are *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Candida krusei*, *Saccharomyces bisporus*, *Schizosaccharomyces pombe* and *Pichia membranifaciens* (Steels et al., 2000; Tribst et al., 2009). They are resistant to 400 mg/ml sorbic acid and up to 900 mg/l benzoic acid at pH 4.0. Their importance in the beverage industry is enhanced by their xerophilic behaviour.

Yeast resistance to preservatives appears to result primarily from an inducible system, which pumps preservatives out of the cell (Virgilio, 2000). It has been reported to involve ejection of protons via the plasma membrane H^+-ATPase proton pump, encoded by PMA1 (Stratford et al., 2013a). This removes protons from the cytoplasm with a normal stoichiometry of 1 proton ejected/ATP although this may decline to 0.1 proton/ATP in

starved cells. In addition, it has been demonstrated in *S. cerevisiae* that Pdr12p which encodes an ABC transporter involved in weak-organic acid resistance has a major effect on weak-acid resistance (Hazelwood et al., 2006). It has been proposed that this plasma-membrane pleiotropic drug resistance pump causes ejection of preservative anions from the cytoplasm into the external media (Stratford et al., 2013a).

The remarkably high resistance of the food spoilage yeast species *Zygosaccharomyces bailii* to weak acid food preservatives such as acetic acid, benzoic acid and sorbic acid was related to its ability to use weak acids as carbon sources, even when glucose is present (Mira et al., 2010). Stratford et al. (2013b) confirmed the high resistance of all 38 tested strains of *Z. bailii* to weak-acid preservatives. Further tests showed that a representative strain of *Z. bailii* was resistant to a wide variety of lipophilic and hydrophilic weak acids. Martorell et al. (2007) also found that *Z. bailii* and *Z. rouxii* displayed physiological behaviours which included resistance to weak-acid preservatives.

2.8.2 Application of disinfectants against yeast formed biofilms

Areas such as industrial water systems and food processing industries are highly affected by problems associated with biofilm formation (Augustin and Ali-Vehmas, 2004; Srey et al., 2013). Spoilage yeasts also proliferate on process surfaces as complexes of yeast cells in biofilms which occur at both low and elevated temperatures (Salo and Wirtanen, 2005) and being protected against sanitizing agents. Colonization of these suspended yeast cells in biofilm growth is generally controlled through cleaning and disinfection. In industrial settings, cleaning and disinfection plans are applied on a regular basis (Araújo et al., 2013). The control of microbial contamination in food processing plants, generally involves clean-in-place (CIP) procedures which consist of running alternated cycles of detergent and disinfectant solutions with water rinses in high turbulence regimes through the plant and pipeline circuits without dismantling or opening the equipment.

Characteristics of an ideal disinfectant include having a broad spectrum of antimicrobial activity, but should be non-toxic and non-irritating (Mazzola et al., 2009). Furthermore, it should be compatible with the surfaces to be disinfected, easy to prepare and use, and should lack any unpleasant odour. Additionally the disinfectant should be stable, affordable and readily available. Formulation of the product may greatly affect the efficacy of antimicrobial active ingredients, such that products with the same levels of antimicrobials may exhibit varying levels of effectiveness. Levels of pH, detergent base, emollients, humectants, ionic nature of the formulation and type of surfactants may affect efficacy (Augustin and Ali-Vehmas, 2004).

According to Brugnoni et al. (2012), the combined action of 0.5% NaOH and 500 ppm sodium hypochlorite produced a reduction of viable cells greater than 70% for *Candida krusei.* Minimum inhibitory concentration studies have shown that quaternary ammonium compounds were effective in reducing *Zygosaccharomyces* spp. in all in-use concentrations tested and *Rhodotorula mucilaginosa* only in the stronger in-use concentration. Hypochlorite, peracetic and phosphoric acid as well as anionic compounds efficiently killed two unknown yeast strains together with *Saccharomyces cerevisiae* isolated from orange juice (Salo and Wirtaten, 2005). Citric and lactic acid compounds were found to be less effective.

The food industry commonly uses disinfectants such as chlorine compounds, alcohols, hydrogen peroxide and peracetic acid compounds, persulphates, quaternary ammonium and iodophors (Salo and Wirtanen, 2005; Bilska, 2014). It is desirable to use a lengthy cleaning procedure in the food industry since the duration of treatment is one of the important factors affecting the antimicrobial activity (Augustin and Ali-Vehmas, 2004), although this may negatively impact production cycles. There is a trend towards longer production runs with shorter intervals for disinfection and cleaning in the food industry. In addition, contact time is not the only aspect but also the concentration and the composition of the agent affect the antimicrobial activity.

2.8.3 Thermal pasteurization

Thermal processing remains the most widely used technology for pasteurization of fruit juice (Shaheer et al., 2014). Juice pasteurization is based on a 5-log reduction of the most resistant microorganisms of public health significance (FDA, 2001). This method relies on the heat that is generated outside a food and then transferred into the food through conduction and convection mechanisms (Pereira et al., 2010). The aim of thermal pasteurization is to kill pathogens and substantially reduce the number of spoilage microorganisms through a suitable time/temperature combination (Ramaswamy et al., 2005). Traditional thermal pasteurization can be classified into low temperature/long-time (LTLT) and high-temperature/short-time (HTST) processes. LTLT pasteurization involves heating a food at about 63^{o}C for no less than 30 min, while for fruit juices, HTST pasteurization is applied at temperatures around 72^{o}C with holding times of 15 s and above (FDA, 2001). Both methods may degrade the taste, colour, flavour and nutritional quality of foods (Charles-Rodr´ıguez et al., 2007). The temperature of HTST pasteurization applied for different fruit juices ranged from 72 to 108^{o}C, while the treatment duration was always 1 min or a shorter time period.

The efficacy of HTST treatment can also be affected by other factors such as the complexity of the product and microorganisms (Chen et al., 2013). HTST pasteurization shows much better performance on less complex single juices than on more complex/viscous multiple-juice products. Single species of microorganisms were more sensitive to heat treatment compared with mixed native populations of microorganisms present in the product.

2.8.4 High pressure processing (HPP)

High pressure processing (HPP) is carried out with intense pressure in the range of 100-1000 MPa, with or without heat, allowing most foods to be preserved with minimal effect (Yordanov and Angelova, 2010). The inactivation of pathogens, spoilage bacteria, yeasts and moulds by HPP has been reported (Olivier, 2010). High pressure processing was demonstrated to be used for inactivation of microorganisms, modification of

biopolymers (enzyme activation or inactivation, protein denaturation, gel formation), preservation of quality attributes (flavour and colour) and functionality (Yaldagard et al., 2008). The mechanism of the system depends on the protein denaturation and cell injury. The enzymes essential in the cell metabolism are denatured after the pressure treatment (Rivalain et al., 2010). Alternatively, shrinkage may occur in the cell size due to the pressure effect. Consequently, membrane construction can be injured or disrupted causing leakage of the cell content (Lado and Yousef, 2002). This brings about cell death (Guerero-Beltran, et al., 2005). Raso, et al. (1998) achieved almost 5-log reductions in the number of *Zygosaccharomyces bailii* after high pressure application of 300 MPa.

HPP (High Pressure Processing) is proven to meet the FDA requirement of a 5-log reduction of microorganisms in fruit juices and beverages without sacrificing the sensory and nutritional attributes of fresh fruits (San Martin et al., 2002). Jams, fruit compotes and fruit dressings have been the first marketed HPP pasteurized foods. These are particularly favourable matrices as the low pH values enhance the HPP microbial inactivation and the chilled storage of acidic foods causes a further inactivation of sub-lethally injured cells (Linton et al., 1999). Recently, such a technique has been successfully used on apple, apricot, cherry and orange juices as well as smoothies (Patras et al., 2009; Keenan et al., 2012). Compared to thermal processing, HPP has many advantages. It can provide safe products with reduced processing time. Moreover, it is environmentally friendly since it requires only electrical energy and no waste by-products generated (Ramaswamy, et al., 2005; Toepfl et al., 2006). Due to these advantages, HPP has been widely used in food product preservation including fruit and beverages in the areas of microbial inactivation and shelf-life extension. However, costs can be high for smaller manufacturers as HPP equipment requires a substantial capital investment.

2.8.5 Ultraviolet Technology (UV)

Ultraviolet technology (UV) has been utilized in the food industry to disinfect water and effectively destroy microorganisms on surfaces and packaging (Chia et al., 2012).

Ultraviolet radiation involves the use of radiation from electromagnetic spectrum from 100 to 400 nm. It is classified as UV-A (320-400 nm), UV-B (280-320 nm) and UV-C (200-280 nm) (Keyser et al., 2008). UV treatment is performed at a low temperature and 254 nm wavelength UV light is widely used in the juice and beverage industry (Rupasinghe et al., 2012). UV-C light inactivates microorganisms by damaging their DNA that absorbs UV light from 200 to 310 nm. UV creates the pyrimidine dimers which prevent microorganisms from replicating and thus rendering them inactive (Keyser et al., 2008). UV radiation was successfully used to reduce the microbial load in different fruit juices, mainly apple juice, fruit nectars and apple cider. Clear liquids such as apple juice needed lower doses of UV to reach efficient reduction of microbial load. Other food liquids such as orange juice with pulp and nectars needed higher doses of UV light due to the greater amount of suspended solids and fibres, which protect microorganisms against the action of UV light (Turtoi and Borda, 2013).

2.8.6 Hurdle Technology

Hurdle technology was developed several years ago as a concept for the production of safe, stable, nutritious, tasty and economical foods (Leistner and Gorris, 1995; Alzamora et al., 2011). Existing and new preservation techniques are deliberately combined to create a series of preservative factors (hurdles) that any microorganism present should overcome. In the last decade, emerging preservation procedures have been included as hurdles in combined preservation systems to ensure food safety and to retain or improve food quality (Alzamora et al., 2011; Shalini and Singh, 2014). This type of processing techniques for food preservation reduce microbial load and at the same time, allow better retention of product flavour, texture, colour and nutrient content than comparable conventional treatments.

There is a wide range of novel alternative physical agents intensely investigated in the last 25 years, which can cause inactivation of microorganisms at ambient or sub lethal temperature (i.e. high hydrostatic pressure, pulsed electric fields, ultrasound, pulsed light and ultraviolet light) (Ferrario et al., 2013). The choice of non-thermal hurdles involved in the combined processes depends on the target within the microbial cells

such as cell membrane, DNA or enzymes system. Other targets involve the extrinsic environment such as pH, temperature or water activity.

Ultrasound (US) has been identified as a potential pasteurization technology for juice that meets the FDA requirement of a 5-log reduction (Patil et al., 2009). According to Butz and Tauscher (2002), it has been shown that ultrasonic waves cause cell rupture attributed to intracellular cavitation. However, ultrasound alone is not very effective for microbial inactivation; it needs to be combined with other technologies to enhance the lethal effect on microorganisms. Ultrasound combined with mild temperature (thermo-sonication) or pressure (mano-sonication) could be an efficient technology to inactivate microorganisms (Ortuño et al., 2012). Several studies have reported reduced microbial counts using ultrasound combined with mild temperature applied on juice. It has also been demonstrated that the use of US combined with thermal temperatures was a promising alternative for inactivating yeasts in apple juice (Ferrario et al., 2013; Abid et al., 2014).

2.9 Routine microbiological monitoring in food production facilities

There are different methods or techniques to ensure quality control in bottling factories. These involve sampling for microbiological analysis. The sampling regime in a factory is influenced by legislation or requirements from specific dealer owned brands. Enumeration of total counts of bacteria, coliforms, yeasts and moulds are the most common microbiological inspections carried out to assess the microbial contamination of surfaces. This type of test will determine the hygiene of equipment and the results are checked for compliancy with standards. South African legislation (R 962 of 2012: Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972) stipulates that swabs should be taken according to the SABS Standard Test method 763: Efficacy of Cleaning Plant, Equipment and Utensils. Upon analysis, viable counts should not be more than 100 CFU/cm^2. Moreover, hand swabs of production workers should be taken every week and examined for the presence of contaminants. When the microbial counts are out of specifications, corrective actions should be implemented and the areas are re-swabbed.

According to South African regulations (R 1111 of 2005: Foodstuffs, Cosmetics and Disinfectants Act, No. 54 1972), 1 ml of fruit juice should not contain a total viable count of more than 10 000 of colony forming units (CFU), a coliform count of more than 100 CFU and a yeast/mould count of 1000 CFU. Samples of the final product and raw materials are tested to maintain quality of products and ensure compliancy with legal requirements. The final products are sampled before the batch is released to retailers.

2.10 Identification Techniques

Traditionally, identification and characterization of yeast species have been based on morphological traits and physiological capabilities (Arias et al., 2002). These characteristics are strongly influenced by culture conditions and can give uncertain results (Guillamón et al., 1998). This conventional methodology requires the evaluation of some 60 to 90 tests, resulting in a complex, laborious and time-consuming process. In addition, conventional methodologies are not suited to industrial laboratories even when these procedures are automated and computerized (Sancho et al., 2000). In contrast, molecular biology techniques provide alternative and additional methods and are becoming an important tool in solving industrial problems (Guillamón et al., 1998). The simplicity of PCR-based detection methods and the highly specific nature of the results has an advantage over most conventional differential methods known today (Sancho et al., 2000).

2.10.1 Molecular analysis

2.10.1.1 Restriction Fragment Length Polymorphism

One of the most successful methods for yeast species identification is restriction fragment length polymorphism (RFLP) analysis of the 5.8S rRNA gene and the two flanking internal transcribed sequences (ITS) (Arias et al., 2002). The fungal rRNA gene consists of the 18S, ITS1, 5.8S, ITS2, 26S, intergenic spacer (IGS) 1, 5S and IGS2

regions (Figure 2.1) (Sugita and Nishikawa, 2003). These genes encoding ribosomal RNAs are organized in arrays which contain repetitive transcriptional units (Korabecna, 2007). The units are transcribed by RNA polymerase I and separated by non-transcribed intergenic spacers (IGS) as represented in Figure 2.1. Because ribosomal regions evolve in a concerted fashion they have low intraspecific polymorphism and high intraspecific variability. Consequently, RFLP analysis of the 5.8S-ITS region is an effective tool for yeast identification.

PCR-RFLP analyses have several advantages that are attractive to quality assurance analysis in the food and beverage industries (Beh et al., 2006). Once a pure yeast culture has been obtained, identification to species level can be done in several hours. Essentially, DNA is extracted from the yeast biomass, amplified by specific PCR using conserved oligonucleotide primers against the 26S and 18S rRNA genes, amplicons digested with the restriction nucleases and the products separated by gel electrophoresis. The work load and equipment needs are minimal and data are generally reproducible, while the expenses and time for sequencing are avoided (Arias et al., 2002).

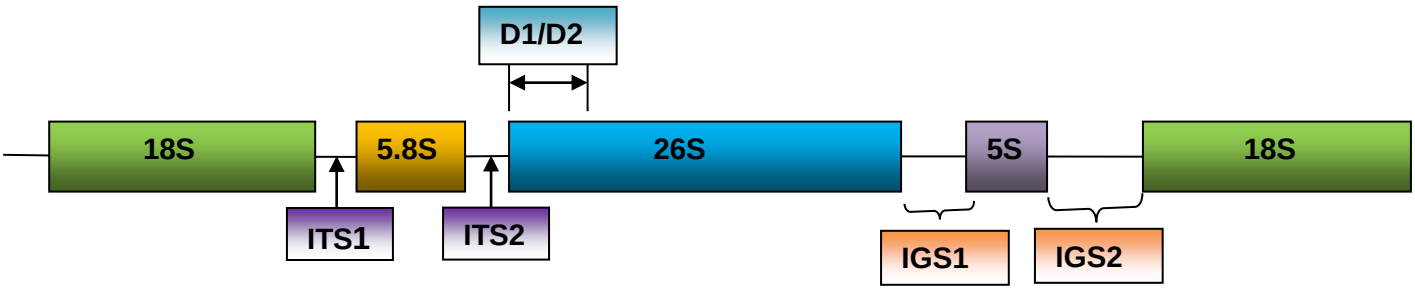

Figure 2.1 Schematic representation of the fungal rRNA gene (Sugita and Nishikawa, 2003).

2.10.1.2 D1/D2 domain sequencing

Comparison of RNA (rRNA) and its template ribosomal DNA (rDNA) has been used extensively in recent years to assess both close and distant relationships among many kinds of organisms including yeast species identification. Sequence-based DNA identification methods are based on sequence analysis, primarily of the 26S rDNA D1/D2 domain and of the 18S subunit (Querol et al., 2003). The use of two universal and two species-specific primers derived from the D1/D2 region of the 26S rDNA and subsequent sequencing of this domain has facilitated rapid and accurate species identification. *Saccharomyces* spp., *Kluyveromyces* spp. and a small collection of wine yeast species have been identified using this method (Hesham et al., 2014).

Kurtzman and Robnett (1998) have shown that most ascomycetous yeast species can be identified from sequence divergence in this domain and that the D1/D2 domain is sufficient to infer phylogenetic relationships between species. The D1/D2 domain sequences for most basidiomycetous yeast species have become available, which means that this sequence is available for almost all currently described yeast species (Fell et al., 2000). The D1 and D2 domains are approximately located in the first 650 bases of the 26S rRNA gene and are two rapidly evolving regions that account for most of the sequence divergence amongst 26S rDNA sequences (Wesselink et al., 2002). Therefore, the D1/D2 domains were used to search for deterministic patterns for yeast species. Analysis of D1/D2 26S rDNA sequences has the advantage that it not only enables species identification, but also permits phylogenetic analysis (Sugita and Nishikawa, 2003). It is very difficult to deduce the phylogenetic position of a genus or family of an isolate from the ITS regions as they are more diverse than the 26S region.

2.10.2 Fourier Transform Infrared Spectroscopy (FT-IR)

FT-IR is a powerful technique for identifying types of chemical bonds in a molecule (Santos et al., 2010). Characterizing the chemical composition of very complex probes such as microorganisms is also a feature performed by this technique. One of the strengths of FT-IR spectroscopy is its ability, as an analytical technique, to obtain

spectra from a very wide range of different compounds. The infrared region of the electromagnetic spectrum extends from the visible to the microwave. This technique has been successfully applied in various fields of quality control and for the identification of filamentous fungi and yeasts (Kummerle et al., 1998; Wenning, 2002; Sandt et al., 2003). Microbiologic FT-IR typing is fast, effective and reagent-free. Moreover, it is applicable to all microorganisms and requires a small quantity of biomass (Essendoubi et al., 2007).

FT-IR spectroscopy has demonstrated its powerful characteristics as a sound technique applied to identification, characterization and authentication of several filamentous fungi and yeast strains (Santos et al., 2010). The advantages of this new approach as a microbial authentication method are: (a) a simple sample preparation procedure, (b) a short time of analysis; and (c) reliability of the data. Statistically, the reference spectrum library is crucial for accurate microbial characterization. It should be assembled based on well characterized strains and species. The success of the method is therefore directly dependent on the complexity of the reference spectrum library (Essendoubi et al., 2007). Identification is limited only by the quality of the reference spectrum library which can be improved steadily by adding further microorganism isolates to the database.

2.10.3 Matrix–Assisted Laser Desorption/Ionisation (MALDI)

Numerous studies demonstrated, that matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) based identification is a rapid and reliable method for routine identification of bacteria, yeast and fungi from clinical samples, even to the subspecies level (Pavlovic et al., 2014). The technique relies on the generation of microorganism protein fingerprints that are compared to reference spectra in a well-characterized library (Dhiman et al., 2011).

MALDI-TOF MS detects many different biomolecules, such as nucleic acids, peptides, proteins, sugars and small molecules (Pavlovic et al., 2013). This technology identifies microorganisms via the generation of fingerprints of highly abundant proteins followed

by correlation to reference spectra in a database. The basic principle of all mass spectrometric methods is the ionization of a neutral molecule and the subsequent accurate determination of the resulting primary ions and their decay products in high vacuum (Parker et al., 2010). A typical mass spectrometer is composed of three components: an ion source, a mass analyser, and the detector.

Until now, not much data has been available concerning the performance of MALDI-TOF MS for classification or identification of foodborne yeast isolates (Pavlovic et al., 2014). Since this technique has been substantially used for clinical isolates, further update of MALDI-TOF MS databases with attention to food relevant yeast species will decrease the portion of not identifiable isolates.

2.11 References

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Chapter 3

Yeast diversity in a typical fruit juice bottling factory

3.1 Introduction

Yeasts are defined as unicellular fungi reproducing by budding or fission (Kurtzman et al., 2011). They are a phylogenetically diverse group of fungi which are divided into two groups, namely, the Ascomycetes and Basidiomycetes (Takashima et al., 2012). The distinction of yeasts from other fungi is based on their sexual spores produced without fruiting bodies. This is one group of microorganisms that is best known for their positive contributions in fermentation of a broad range of food commodities such as bread, alcoholic beverages and other products (Fleet, 1992). However, yeasts also play a role as spoilage organisms in foods and beverages (Makino et al., 2010).

Yeasts are usually the contaminants that affect the quality and shelf life of fruit juices (Sancho et al., 2000; Arias et al., 2002; Maciel et al., 2013). This contamination represents a great problem to industries that process fruit or fruit products (Patrignani et al., 2010). This negative effect is linked with well-known physiological characteristics of yeasts (Makino et al., 2010). The low pH and high sugar content of the product favour yeast growth and consequently product deterioration is predominantly due to yeast activity (Patrignani et al., 2010). Yeasts are characterized by a wide dispersion of natural habitats, which are essential vehicles for carrying yeasts into food-processing facilities. They flourish on flowers, plant leaves and especially fruits (Deak and Beuchat, 1996; Lachance et al., 2001). They also occur on the skin, hide, feathers and the alimentary tract of herbivorous animals. Some of these yeasts are associated with insects and several are part of the normal intestinal microflora of humans. Soil is an important reservoir in which yeasts can survive during unfavourable conditions (e.g. extreme pH, temperatures and low nutrients) and then be dispersed to foods (Deak and Beuchat, 1996; Polyakova et al., 2001; Botha, 2011).

The development of yeast biofilms may cause adverse effects on processing equipment in factories (Brugnoni et al., 2007). While previous studies on biofilm development has been devoted to bacterial species, there is little information about yeast diversity or their adhesion capacity in fruit juice processing lines (Brugnoni et al., 2012). The microorganisms involved are likely to be a mixture of many species in a processing environment (Bagge-Ravn, 2003). In food processing lines, yeasts belonging to the genera *Saccharomyces*, *Candida* and *Rhodotorula* have been isolated from conveyor track surfaces as well as can and bottle warmers in the packaging departments of the beverage industry (Brugnoni et al., 2012). Knowledge of the diversity of microorganisms in and on process equipment is regarded as valuable information in designing cleaning and disinfection procedures (Bagge-Ravn, 2003).

The ability to control contamination of food products and to design adequate intervention measures requires an appropriate strategy which includes hygiene measures. Disinfection and sanitizing through CIP (Cleaning in place) and the addition of preservatives to fruit juice, are the two methods used by this fruit juice facility to inhibit the growth of yeasts. Analysis of critical control points of production lines and investigations of the routes and sources of contamination are also essential to decrease and eliminate spoilage microorganisms (Lopandic et al., 2006). Microbial monitoring in bottling factories involves the analysis of pulps, water, air and equipment. Enumeration of microorganisms on selective media is the most common method in practice, which for yeast and mould only provide total counts (Combina et al., 2008). When planning approaches and systems to prevent spoilage it is ideal to be familiar with the identity of spoilage microorganisms present in the product to get an insight into the source of contamination (Loureiro, 2000; Combina et al., 2008). Therefore, this investigation aimed to isolate and identify yeasts associated with the production environment and equipment of this fruit juice bottling facility.

3.2 Materials and Methods

3.2.1 Sampling protocol

Samples were obtained from a fruit juice bottling factory in Bloemfontein, South Africa. The factory has been operating for 24 years producing fruit juice concentrates of different flavours. It consists of nine blending tanks and three filling lines. Approximately 24 000 litres of juice is produced a day.

Surface swabs were obtained from the production environment and processing equipment. Areas included the refrigerator, powder blenders, pipes, blending tanks, holding tanks, nozzles and the ramp (Figure 3.1). Yeast isolates originating from weekly routine analysis of surface swabs and air samples taken after Cleaning in Place (CIP) protocols were carried out, were also isolated from Chloramphenicol agar plates. All isolates were collected over a period of one year and cryopreserved in 15% glycerol at -20°C.

3.2.2 Enumeration and isolation of yeasts

Surface swabs were suspended in 10 ml peptone water (Merck), vortexed for 1 min and serially diluted. Dilutions were plated onto Rose Bengal Chloramphenicol (RBC) agar (Merck) and incubated at 30°C for 48 h. Yeast colonies from the Chloramphenicol agar plates, which were provided by the factory technician, were also transferred to RBC agar and incubated at 30°C for 48 h. The resulting colonies were selected based on colony morphology differences and purified by repeated sub-culturing (Barata et al., 2008).

3.2.3 Yeast identification

3.2.3.1 PCR amplification and RFLP analysis of 5.8S-ITS rDNA

Whole cell PCR amplification and RFLP analysis of the 5.8S-ITS rDNA region were performed on all isolates and reference strains. For comparison and preliminary

identification, 17 reference strains frequently isolated from fruit juice were obtained from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection of the University of the Free State (Table 3.1).

Yeast cells from 48 h single colonies were suspended in 50 µl PCR reaction mix containing 0.52 µM primer ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), 0.2 µM dNTPs, 1X reaction buffer Thermopol® (New England Biolabs), and 1 U of NEB Taq Thermopol® (New England Biolabs). Amplification conditions included 1 cycle at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 1 min. A final elongation step was performed at 68°C for 7 min. Successful amplification was confirmed by agarose gel (1%) electrophoresis. PCR products were stained with ethidium bromide (Merck) and visualised under UV light. These PCR products represent the 5.8S – ITS rRNA gene and varied in length from 300 – 900 bp. All amplicons (10 ul) were digested with 1 unit of *Cfo*I, *Hae*III and *Hinf*I restriction enzymes (Thermo Scientific) using FastDigest™ and Tango™ buffers (Thermo Scientific) in separate reactions (Esteve-Zarzoso et al., 1999). Fragments were separated in a 3% agarose gel and stained and visualised as mentioned. Digital images were captured with the Molecular Imager® Gel Doc™ XR system (BioRad laboratories Inc.). Band sizes were calculated with reference to a GeneRuler™ 1 kb DNA ladder Plus and GeneRuler™ 50 bp DNA ladder (Thermo Scientific) using Quantity One® 1-D Analysis software (BioRad laboratories Inc.). Resulting PCR-RFLPs were grouped according to profiles (Table 3.2), compared to the reference strain profiles for preliminary identification and Sanger sequenced for confirmation.

Table 3.1 List of reference strains

Yeast species	Strain no.
Candida intermedia	UOFS Y-0649
Candida parapsilosis	UOFS Y-0206
Candida tropicalis	UOFS Y-0534
Dekkera anomala	UOFS Y-1062
Hanseniaspora occidentalis	UOFS Y-0153
Kluyveromyces marxianus	UOFS Y-0797
Lodderomyces elongisporus	UOFS Y-2394
Millerozyma farinosa	UOFS Y-0203
Pichia kudriavzevii	UOFS Y-0814
Rhodotorula slooffiae/Cystobasidium slooffiae	UOFS Y-0972
Saccharomyces bayanus	UOFS Y-0912
Saccharomyces cerevisiae	UOFS Y-0792
Saccharomycodes ludwigii	UOFS Y-0540
Torulaspora delbrueckii	UOFS Y-1016
Wickerhamomyces anomalus	UOFS Y-0810
Zygosaccharomyces bailii	UOFS Y-1535
Zygosaccharomyces rouxii	UOFS Y-0763

3.2.3.2 Yeast genomic DNA extraction

Genomic DNA (gDNA) extraction was performed on the representative isolates from the different profiles as described by Labuschagne and Albertyn (2007). The isolates were cultivated on 5 ml Yeast extract-malt glucose (YM) broth in 16 mm capped test tubes at 30ºC for 48 h while shaking. Cells were harvested by centrifugation following addition of 500 µl DNA lysis buffer and glass beads. This solution was vigorously mixed and cooled

on ice for 5 min. Ammonium acetate (275 ml, 7M, pH 7.0) was added. After incubation at 65°C for 5 min followed by 5 min on ice, 500 µl chloroform was added, vortexed and centrifuged (13 000 rpm, 2 min at 25°C). The supernatant was transferred to a new tube and DNA was precipitated with isopropanol and centrifuged at 11 000 rpm for 2 min at 4ºC. The pellet was washed with 70% (v/v) ethanol, dried and dissolved in 100 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8.0).

3.2.3.3 Amplification and sequencing of D1/D2 domain

The D1/D2 domain of the 26S rRNA gene was amplified from 3 isolates representative of a specific PCR-RFLP profile and sequenced for identity confirmation. One ul of extracted gDNA was used as template in a 25 µl PCR reaction containing 0.52 µM primer NL 1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL 4 (5'GGTCCGTGTTTCAAGACGG-3') (White et al., 1990), 0.2 µM dNTPs, 1X reaction buffer Thermopol® (New England Biolabs), and 1 U of NEB Taq Thermopol® (New England Biolabs). Amplification conditions included 1 cycle at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 1 min. A final elongation step was performed at 68°C for 7 min. Successful amplification was confirmed by agarose gel (1%) electrophoresis. PCR products were stained with ethidium bromide (Merck) and visualised under UV light.

Sequencing was performed on the ABI Prism 3130 XL genetic analyser using the Big Dye® Terminator V3.1 Cycle Sequencing Kit and DNA was precipitated with EDTA and ethanol (Applied Biosystems). Both strands of amplified DNA were sequenced, using primers NL-1 and NL-4 in separate reactions, to eliminate sequencing artefacts and to ensure accuracy of data generated. The DNA Baser (v4) sequence assembly software was used to assemble DNA contigs. The sequences obtained from the contigs were compared to those present in the National Centre for Biotechnology Information (NCBI) database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the BLAST algorithm (megablast) (Altschul et al., 1997) for identification. Sequences were deposited into the NCBI database and accession numbers are depicted in Table 3.3.

3.3 Results and discussion

Figure 3.1 represents the floor plan of the production and processing areas in the factory. The production flow is as follows: The pulp from the refrigerator (area A) and dry powder ingredients (area B) delivered by powder blenders and mixed in blending tanks (area C). Juice is then pumped in to the holding tanks (area E) and filled into bottles on the filling line (area F). The raw materials store is separate from the other sections, bottle caps are delivered from the second floor above (area E and F) and no barriers exist among the different sections of production and processing. (Area G) is not part of the workflow but serves as an entry site for workers into the factory.

3.3.1 PCR and RFLP analysis

A total of 201 yeasts were isolated and identified according to 5.8S-ITS polymorphisms (White et al., 1990). The isolates showed different PCR product sizes, ranging from 300 to 900 bp (Figure 3.2, Table 3.2). The PCR products digested with *Cfo*I, *Hae*III and *Hinf*I enzymes were analysed for all isolated strains and 18 distinct profiles were obtained (Table 3.2), designated by an alphabet letter. For comparison, PCR-RFLP of the 5.8S-ITS region was simultaneously applied to reference strains from the UNESCO-MIRCEN Biotechnological Yeast Culture Collection as controls (Table 3.1). Fragments smaller than 50 bp were not included in Table 3.2. Representatives of each profile were confirmed by sequencing the D1/D2 domain of the 26S rRNA gene (Table 3.3).

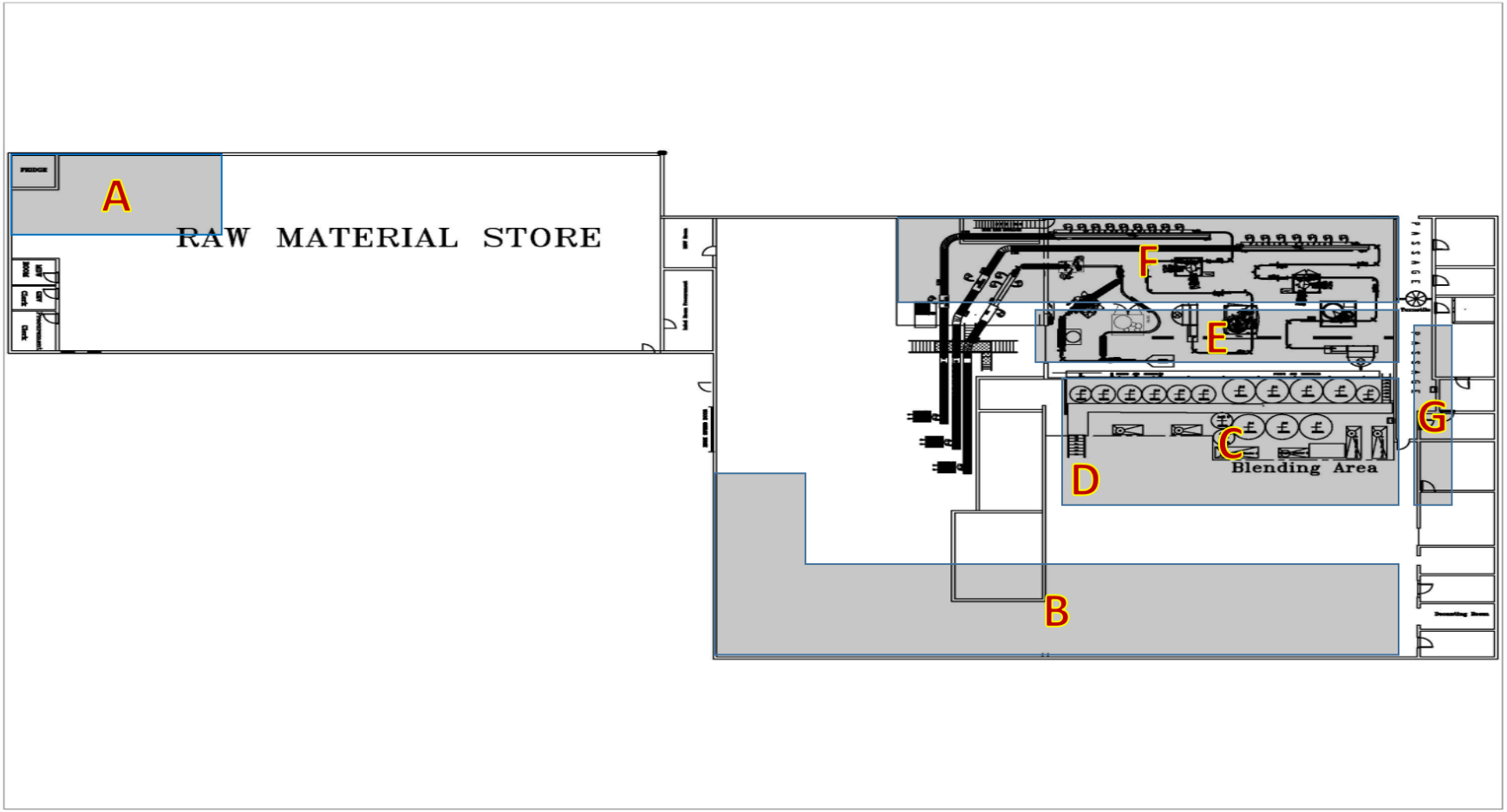

Figure 3.1 Schematic representation of the factory layout showing different production and processing areas: A– Refrigerator, B– Dry raw materials, C– Powder blenders, blending tanks and pipes, D– Ramp (Steel stairs), E– Fillers, Holding tanks and nozzles, F– Filling lines, bottles and caps, G– Entrance to production and processing areas.

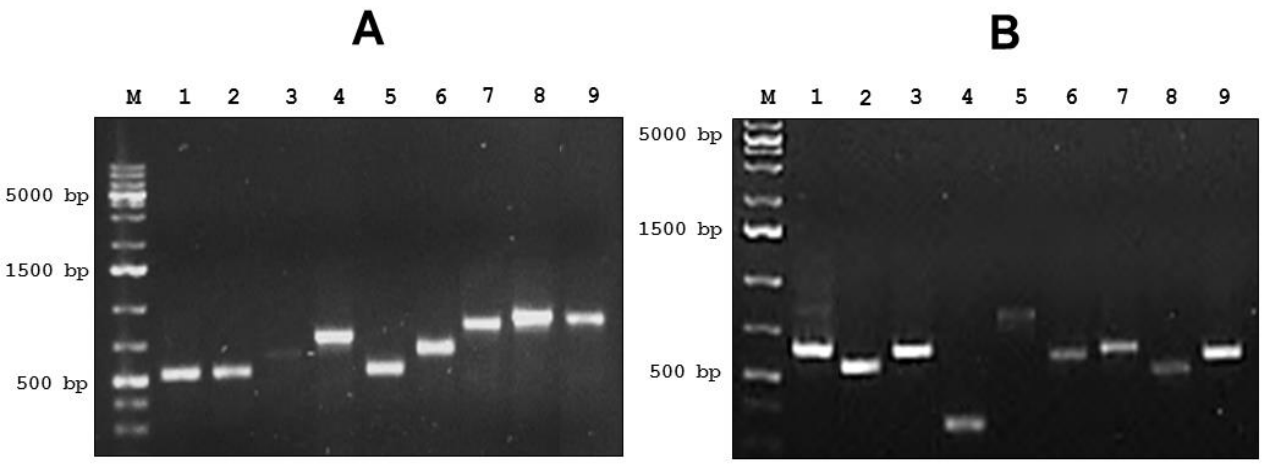

Figure 3.2 1% agarose gel of 5.8S-ITS PCR products representing selected reference strains (A) and unidentified isolates (B). Lane M in both gels represents GeneRuler^{TM} 1 kb Plus DNA Ladder (Thermo Scientific). Gel (A) lane 1- *Dekkera anomala*, 2– *Candida tropicalis*, 3- *Pichia kudriavzevii*, 4- *Kluyveromyces marxianus*, 5- *Candida parapsilosis*, 6- *Wickerhamomyces anomalus*, 7- *Torulaspora delbrueckii*, 8- *Saccharomyces bayanus*, 9- *Saccharomyces cerevisiae*. Gel (B) unidentified isolates representing different profiles; lane 1- X; 2- O; 3- F; 4- G; 5- D; 6- W; 7- A; 8- E and 9- H.

Profile G (*Yarrowia lipolytica*) had the smallest amplicon size of 377 bp, this differentiating it from the rest of the yeasts identified (Table 3.2). Yeast species belonging to profiles G (*Yarrowia lipolytica)*, C1 *(Candida intermedia)* and V (*Trichosporon ovoides)* had smaller amplicon sizes ranging from 377 – 480 bp (Table 3.2). A similar characteristic of these three species was a lack of *Hae*III restriction site and digestion with *Hinf*I resulted in the production of two bands almost equal in size. Profile A1 (*Candida parapsilosis)* and P1 (*Cryptococcus laurentii)* have the same amplicon size but digestion with *Cfo*I differentiates the two species. The data showed that profile R1 *(Candida tropicalis* and *Candida sojae)* could not be differentiated based on 5.8S-ITS PCR amplicon size and restriction profiles, since they exhibit similar bp sizes for all the amplified and digested fragments. Phylogenetic analysis of the nuclear large subunit (26S) ribosomal DNA partial sequences, places *C. tropicalis* near *C. sojae* (Kurtzman and Robnett, 1998). Thus, profile R1 required further processing by sequencing the D1/D2 domain of the 26S rDNA to allow differentiation.

Table 3.2 Characterization of yeast isolates based on 5.8S-ITS rDNA region PCR and RFLP data, as well as D1/D2 domain of the 26S rDNA sequence identifications. Data arranged according to PCR product size

Profile	RFLP based identification (compared to reference strains)	Fragment lengths (bp)				Identification (D1/D2 domain sequencing)
		PCR	*Cfo*I	*Hae*III	*Hinf*I	
G	unknown	377	214, 174	377	182	*Yarrowia lipolytica*
C1	*Candida intermedia* (UOFS Y-0649)	400	212,174	400	215, 193	*Candida intermedia*
V	unknown	480	272	476	234	*Trichosporon ovoides*
A1	*Candida parapsilosis* (UOFS Y-0206)	515	290, 220	410, 112	281, 257	*Candida parapsilosis*
P	unknown	515	252, 202, 64	399	236	*Cryptococcus laurentii*
R1	*Candida tropicalis* (UOFS Y-0534)	527	293, 238	468	274	*Candida sojae*
O	*Lodderomyces elongisporus* (UOFS Y-2394)	576	323, 240	527	298, 261	*Lodderomyces elongisporus*
X	unknown	596	317	423, 141	318	*Candida oleophila*
H	unknown	611	612	586	339, 265	*Candida spandovensis*
A	unknown	612	281, 310	508	357, 280	*Filobasidium capsuligenum*
H	unknown	624	392, 308	487	261, 242, 146	*Filobasidium uniguttulatum*
I1	unknown	634	320, 226, 66	420, 137	312	*Candida quercitrusa*
E	unknown	635	385	536	350, 292	*Cryptococcus saitoi*
F	*Wickerhamomyces anomalus* (UOFS Y-0810)	640	569	640	308	*Wickerhamomyces anomalus*
K1	unknown	643	331	643	343, 171, 122	*Zygoascus hellenicus*
W	*Rhodotorula slooffiae/ Cystobasidium slooffiae* (UOFS Y-0972)	647	647	647	325, 253	*Rhodotorula slooffiae*
R	unknown	687	341, 321	387, 209	221, 215, 103	*Rhodotorula dairenensis*
D	*Zygosaccharomyces bailii* (UOFS Y-1535)	782	336, 284	712	331, 230, 160	*Zygosaccharomyces bailii*

Profile H *(Candida spandovensis)* and A *(Filobasidium capsuligenum)* have similar amplicon sizes and a similar restriction pattern when digested with *Hin*fI but restriction with *Cfo*I and *Hae*III differentiates the two species (Table 3.2). This emphasises the requirement for more than one restriction enzyme to be used to reliably identify a species or genus (Satora et al., 2013). Profile I1 *(Candida quercitrusa)* and E *(Cryptococcus saitoi)* have the same amplicon size but digestion with all three enzymes resulted in different profiles, enabling immediate distinction between the two species. Profile K1 *(Zygoascus hellenicus)* and F *(Wickerhamomyces anomalus)* have the same amplicon size and profile when digested with *Hae*III, but digestion with *Cfo*I and *Hinf*I reveals different profiles for both. The restriction enzyme *Hae*III showed one fragment of 640 bp for the isolates belonging to *Wickerhamomyces anomalus* (Table 3.2). The enzyme *Hinf*I showed a single restriction site resulting in two fragments of the same size (308 bp) presented as a single band on the agarose gel. The pattern is in accordance with the results reported by Jeyaram et al. (2008) and Pham et al. (2011). Profile K1 *(Zygoascus hellenicus)* similarly lacks a restriction site with *Hae*III (Sun and Liu, 2014).

Yeasts belonging to profile D (*Zygosaccharomyces bailii*) had the largest amplicon size of 782 bp. This characteristic distinguishes this species immediately from the rest of the yeast species identified in this study (Table 3.2). Amplicon sizes for profiles O, X, H, W, and R ranged from 527 – 687 bp. These species had unique profiles and included *Lodderomyces elongisporus, Candida oleophila*, *Filobasidium uniguttulatum*, *Rhodotorula slooffiae/Cystobasidium slooffiae* and *Rhodotorula dairenensis,* respectively.

Minor discrepancies were observed in amplicon sizes and restriction profiles among species in different studies. Small differences in the fragment sizes may be related to sequence differences between strains of a given species. It may be expected that differences in recorded fragment sizes as great as 20 bp are possible simply due to the manner in which the bands sizes were determined and this would account for many of the small size variations reported by different researchers for the same strain of a species (Esteve-Zarzoso et al., 1999; Granchi et al., 1999; Coton et al., 2006; Pham et al., 2011).

3.3.2 Sequences of the D1/D2 region of the 26S rDNA

Eighteen profiles were obtained from the RFLP data and three representatives of each were identified using D1/D2 domain sequencing. D1/D2 domain rDNA PCR products ranged from 500 to 600 bp (Figure 3.3) as expected (Kurtzman and Robnett, 1998). Sequence comparisons were performed for all yeasts using the Basic Local Alignment Search Tool (BLAST) program within the GenBank database. Each profile was ascribed to the species showing the highest matched sequence identity (Table 3.3). Sequence *E*-values of all isolates were 0.0 and the accession numbers of the BLAST hits are shown in brackets and those which were submitted to NCBI are indicated in the designated column.

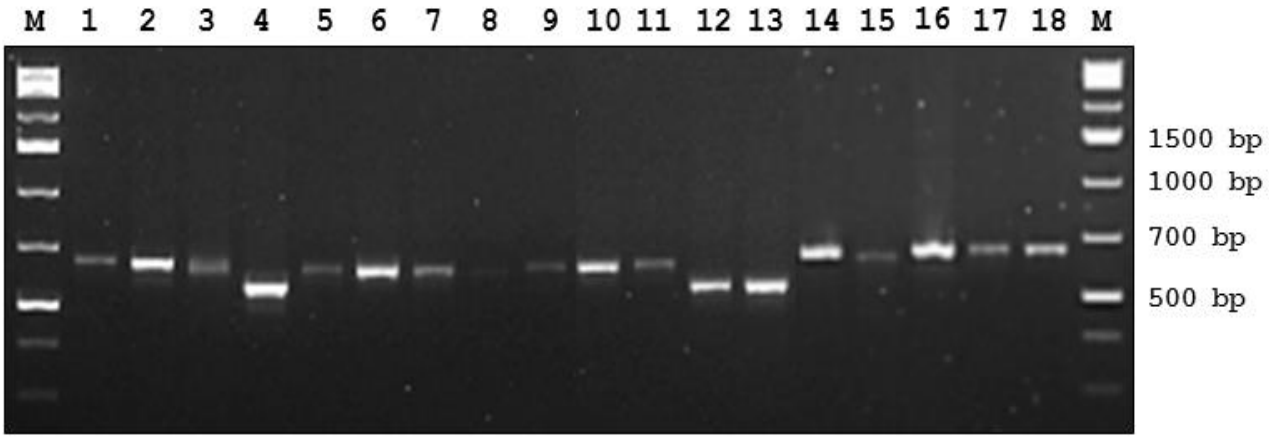

Figure 3.3 1% agarose gel showing D1/D2 domain PCR products of isolates representing the 18 profiles described Tables 3.2 and 3.3. Lanes M-GeneRuler^{TM} 50 bp DNA Ladder (Thermo Scientific). Lanes 1-*Filobasidium capsuligenum,* 2- *Filobasidium uniguttulatum,* 3- *Zygoascus hellenicus,* 4- *Candida intermedia,* 5- *Candida parapsilosis,* 6- *Candida sojae,* 7- *Candida quercitrusa,* 8- *Candida spandovensis,* 9-*Candida oleophila,* 10- *Lodderomyces elongisporus,* 11- *Wickerhamomyces anomalus,* 12- *Yarrowia lipolytica,* 13- *Zygosaccharomyces bailii,* 14-*Cryptococcus laurentii,* 15-*Cryptococcus saitoi,* 16- *Rhodotorula dairenensis,* 17- *Rhodotorula slooffiae,* 18-*Trichosporon ovoides.*

Most of the sequences exhibited sequence identities of 98 – 100% during comparison with sequences available on the NCBI nucleotide database. Only *Cryptococcus laurentii* and *Rhodotorula slooffiae* showed identities of 97%. The preliminary identification with the reference strains successfully identified all isolates which were represented with the exception of *Candida sojae*.

The 5.8S-ITS RFLP profile for each of the yeast species associated with this factory can now be utilized as a control panel for quick analysis and identification of new contamination detected during routine monitoring. Species with unique restriction profiles have been observed and may potentially provide reliable restriction profiles for identification. In one instance, species of the same genus have displayed similar restriction profiles (*Candida tropicalis*/*Candida sojae)* and can thus not be considered species-specific, but necessitates confirmation by sequencing the D1/D2 region of the 26S rDNA for identification.

Table 3.3 Identification of yeast isolates compared and submitted to NCBI database

Profile	Blast hit	# of isolates	Identity	bp	Accession number
	Ascomycetes				
C1	*Candida intermedia* isolate C1/23 large subunit ribosomal RNA gene, partial sequence (KM246051.1)	27	100%	523	KU708236
A1	*Candida parapsilosis* strain N01-1.2 26S ribosomal RNA gene, partial sequence (FJ432627.1)	38	99%	614	KU708237
R1	*Candida sojae* partial 26S rRNA gene, strain IMUFRJ 51946 (FN424102.1)	7	100%	525	KU708238
I1	*Candida quercitrusa* 18S rRNA gene (partial), ITS1, 5.8S rRNA gene, ITS2 and 26S rRNA gene (AM160627.1)	5	100%	587	KU708239
U	*Candida spandovensis* strain NRRL Y-17761 26S ribosomal RNA gene, partial sequence (DQ438228.1)	11	99%	558	KU708240
X	*Candida oleophila* strain 163 26S ribosomal RNA gene, partial sequence (JN544012.1)	8	100%	572	KU708241
O	*Lodderomyces elongisporus*, 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S (LN827703.1)	12	100%	569	KU708243
F	*Wickerhamomyces anomalus* strain Cf20 26S ribosomal RNA gene, partial sequence (KM978209.1)	25	100%	573	KU708244
G	*Yarrowia lipolytica* strain HG12 26S ribosomal RNA gene, partial sequence (JQ680462.1)	6	99%	523	KU708245
D	*Zygosaccharomyces bailii* 26S ribosomal RNA gene, partial sequence (KF908879.1)	6	99%	603	KU708246
K1	*Zygoascus hellenicus* strain CBS 5839 26S large subunit ribosomal RNA gene, partial sequence (AY447007.1)	7	99%	597	KU708235
	Basidiomycetes				
P	*Cryptococcus laurentii* strain HB84-1 26S ribosomal RNA gene, partial sequence (KJ507290.1)	5	97%	624	KU708248
E	*Cryptococcus saitoi* strain HB26-2 26S ribosomal RNA gene, partial sequence (KJ507267.1)	4	100%	607	KU708249
A	*Filobasidium capsuligenum* 26S ribosomal RNA gene, partial sequence (AF075501.1)	14	100%	633	KU708233
H	*Filobasidium uniguttulatum* strain PD411 large subunit ribosomal RNA gene, partial sequence(KJ439613.1)	8	98%	638	KU708234
R	*Rhodotorula dairenensis* strain MB202 26S ribosomal RNA gene, partial sequence (KC798400.1)	6	99%	600	KU708250
W	*Rhodotorula slooffiae* gene for large subunit ribosomal RNA, partial sequence (AB566328.1)	10	97%	630	KU708251
V	*Trichosporon ovoides* partial 26S rRNA gene (HE660084.1)	8	99%	626	KU708252

3.3.3 Yeast diversity in the fruit juice bottling factory

Many types of yeasts are potential spoilage agents of fresh and concentrated fruit juices due to favourable pH conditions and high sugar levels of these beverages (Tribst et al., 2009). Contamination may originate from any step along the beverage manufacturing process (Guillamó et al., 1998). Raw materials, factory environment, packaging and processing equipment are all potential contamination sources (Stratford, 2006). In a 'Forensic approach' to spoilage of soft drinks, Davenport (1996; 1997; 1998) noted that most yeast contaminants encountered could be divided into four categories, i.e. Groups 1-4. Group 1 constitute spoilage yeasts which are fermentative and preservative resistant, Group 2 comprises spoilage or hygiene types and Group 3 are indicators of poor factory hygiene. Group 4 yeasts are 'aliens' which are out of their normal environment.

A total of 201 yeasts belonging to 10 different genera were isolated and identified from the production environment and process equipment. Ascomycetous yeasts included the species from the genera *Candida, Lodderomyces, Wickerhamomyces, Yarrowia*, *Zygosaccharomyces* and *Zygoascus* (Figure 3.4). Yeast diversity in this factory was dominated by Ascomycetes, which was not surprising since most of the ascomycetous yeasts are found in environments with high concentrations of sugar (Van Eck et al., 1993). Basidiomycetous yeasts were represented by *Rhodotorula slooffiae*/*Cystobasidium slooffiae*, *Rhodotorula dairenensis*, *Cryptococcus laurentii*, *Cryptococcus saitoi*, *Filobasidium uniguttulatum*, *Filobasidium capsuligenum* and *Trichosporon ovoides*. Basidiomycetous yeasts are generally found in soil, plant materials and bird droppings and are not usually associated with spoilage and industrial processes, but are regarded as hygiene indicator species (Tokuoka, 1993; Davernport, 1996; Tekolo et al., 2010). As such, these yeasts were also less abundant in the factory equipment compared to ascomycetous yeasts (Figure 3.4).

Candida species made up the largest proportion of yeasts found on all the equipment (Figure 3.4). These species were present on all the equipment, but not in the refrigerator. The genus *Candida* has become one of the largest in species number, present in almost every environment (Frutos et al., 2004). Yeasts of this genus are

abundantly distributed in nature on land and sea as well as being associated with animals, plants or inanimate objects. As observed in this study, species of this genus can also be found in food processing environments and have been recovered as contaminants in a high number of foods, including fruits, fruit juices, soft drinks, alcohol beverages and products with a high sugar content (Frutos et al., 2004; Chang et al., 2012; Maciel et al., 2013).

Candida parapsilosis, Candida intermedia, *Lodderomyces elongisporus*, and *Wickerhamomyces anomalus* are classified as Group 2 yeasts which are able to cause spoilage and are indicators of problems in the cleaning program. The overall yeast distribution shows that *C. parapsilosis* and *L. elongisporus* are widely distributed in this factory, isolated from all other areas tested except the holding tanks, refrigerator and pipes (Figure 3.4). *C. parapsilosis* is an opportunistic spoilage yeast, frequently isolated in low numbers from a variety of sources and causing occasional spoilage in a wide variety of materials (Boekhout and Robert, 2003). These materials range from soft drinks to shampoo. *L. elongisporus* similarly causes occasional spoilage of both soft drinks and fruit juices in bottling plants. Both these species, extensively distributed in nature and dispersed by insects, animals and humans, are potential causes of food and beverage contamination, as well as infectious disease (Chang et al., 2012).

Zygoascus hellenicus and *Zygosaccharomyces bailii* resort under Group 1 spoilage yeasts (Davernport, 1996, 1997, 1998). The occurrence of these yeasts indicates the likely presence of fruit concentrates and sugar syrup residues in the factory environment. *Trichosporon ovoides*, *Cryptococcus laurentii*, *Rhodotorula slooffiae*, *Filobasidium capsuligenum* and *Filobasidium uniguttulatum* fall under Group 3 yeasts. These yeasts indicate problems related to the hygiene status of the factory when isolated in high numbers and also reveals the presence of excessive dust-like particles. *Yarrowia lipolytica* belongs to Group 4 yeasts indicative of the presence of oil or dairy products.

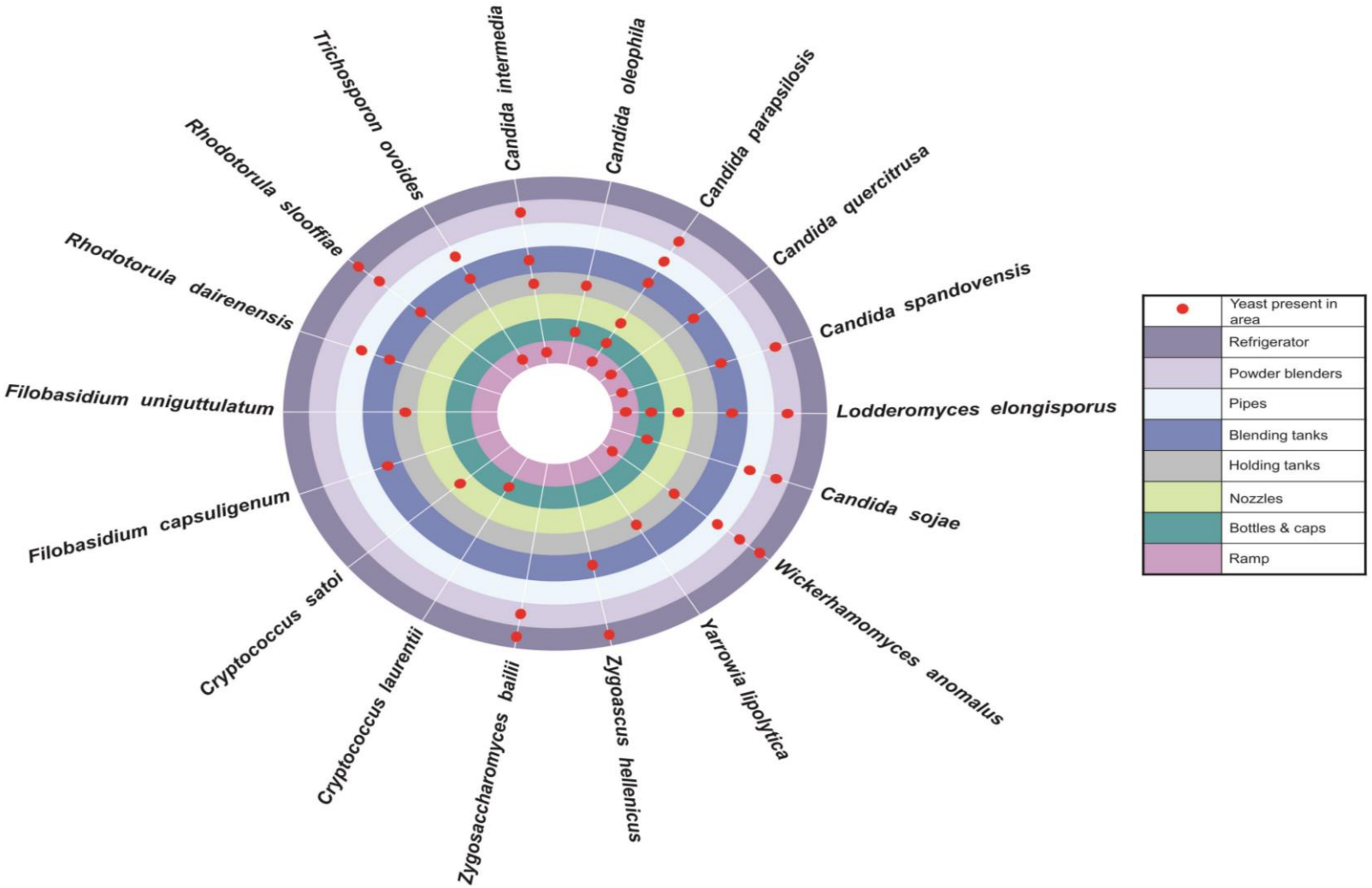

Figure 3.4 Data wheel showing the yeast distribution in the fruit juice bottling factory, in terms of culturability and presence. Isolates originated from the refrigerator (area A), powder blenders, pipes, blending tanks (area C), holding tanks, nozzles (area E), bottles, caps (area F) and the ramp (area D).

The level of yeast contamination varied among the different equipment and diversity findings are discussed based on the workflow in the factory (areas A-F, Figure 3.1). Four types of yeasts namely, *Cystobasidium slooffiae* (61%), *Zygoascus hellenicus* (13%), *Wickerhamomyces anomalus* (13%) and *Zygosaccharomyces bailii* (13%) were isolated from the air samples taken from the refrigerator (area A) where the concentrated pulps are stored. *Cystobasidium slooffiae* previously known as *Rhodotorula slooffiae* (Yurkov et al., 2015) was the dominant yeast isolated from this area. The latter may be due to the reason that basidiomycetous pigmented yeasts such as *Rhodotorula* spread easily in the air (Dworecka-Kaszak and Kizerwetter-Swida, 2011). All of the species isolated from the refrigerator have been frequently isolated from fruits (Thomas and Davenport, 1985; Prakitchaiwattana et al., 2004; Anhansal et al., 2008; James and Stratford, 2011; Walker, 2011).

In the powder blenders (area C) which are used for mixing powder ingredients, eight different yeast species were detected. *Candida intermedia* (34%) and *Candida parapsilosis* (32%) were the dominant yeasts isolated. Both yeasts have previously been isolated from reconstituted fruit juice (Maciel et al., 2013) and *C. parapsilosis* has been reported as an opportunistic pathogen responsible for various mycoses (Jaques and Casaregola, 2008). It is reasonable to assume that contamination of equipment by *C. parapsilosis* is a result of food handlers since isolates of *C. parapsilosis* are frequently found in blood, skin and nails (including hands of health-care workers) (Nosek et al., 2009). Furthermore, Welthagen and Viljoen (1998) reported that workers' hands and aprons were also responsible for a high rate of yeast contamination in other food processing environments. All yeast species isolated from the powder blenders can potentially contaminate the product given that the powder blenders are directly linked to the blending tanks. *Zygosaccharomyces bailii* (2%) was isolated from the powder blenders at a relatively low occurrence compared to *Wickerhamomyces anomalus* (12%) and *Lodderomyces elongisporus* (8%). *L. elongisporus* has been reported to cause occasional spoilage of soft drinks and fruit concentrates (Stratford and James, 2003). *Candida spandovensis* (8%) which has been associated with frozen fruit pulps (Trindade et al., 2002) and *Candida sojae* (2%) was also isolated from the powder blenders. The yeast species isolated from the powder blenders comprises of Group 2 yeasts which are

spoilage and hygiene types, able to cause spoilage of fruit juices, but only if complications arise during manufacturing such as low level or absence of preservative, ingress of oxygen, failure of pasteurization or poor standards of hygiene (Davenport, 1996).

The pipes (area C) which connect the powder blenders to the blending tanks were largely colonised by *Candida parapsilosis* (50%). Species of *Trichosporon ovoides* (17%) was isolated from the pipes and *Trichosporon* spp. are classified by Davenport (1996; 1997; 1998) as belonging to Group 3 organisms which are hygiene indicators, not causing spoilage. *Trichosporon* spp. are widely distributed in nature and found predominantly in tropical and temperate areas (Colombo et al., 2011). These species are able to utilize different carbohydrates and carbon sources and degrade urea, but members of this genus are non-fermentative. *Wickerhamomyces anomalus*, *Candida sojae* and *Rhodotorula dairenensis* were also isolated. Not unexpectedly, the blending tanks shared similar diversity with the pipes and also contained *Filobasidium capsuligenum*, *Candida intermedia*, *Lodderomyces elongisporus*, *Rhodotorula slooffiae*, *Zygoascus hellenicus, Candida quercitrusa* and *Candida spandovensis.* The high sugar content and low water activity of the ingredients in the blending tanks favour the growth of yeasts and this contributed to the large diversity isolated from this equipment.

Wickerhamomyces anomalus (28%) and *Filobasidium uniguttulatum* (28%) were equally dominant in the holding tank (area E). This tank also harboured *Yarrowia lipolytica*, *Candida intermedia* and *Candida oleophila. C. oleophila* is widely distributed in fruits (Glushakova et al., 2007). *Yarrowia lipolytica* is known to occasionally cause spoilage of dairy products (Zinjarde, 2014). The presence of a fruit dairy blend in the tank, such as the Coco Pine Dairy Blend, creates a hydrophobic and substrate-containing environment, which can encourage the growth of *Y. lipolytica.*

The steel stairs used by the production staff to reach the top of the blending tanks are referred to as “the ramp” (area D) and the yeast diversity found here is similar to that of the blending tanks. The workers are continuously walking up to the tanks and it has been found that footwear can be a vehicle for the transfer of microorganisms from production areas. This structure can be a source of yeasts detected in the tanks.

Studies by Taylor et al. (2002) have shown that under factory conditions, when footwear was soiled with both food debris and microorganisms, the foot baths and boot washers were largely ineffective at removing all organic soil and could not remove or decontaminate all microorganisms. In addition, footwear can transport contamination at significant distances (Gardner, 2014). Boot washers also have the potential to disperse microbial aerosols that can transfer contamination from the footwear to the operative's clothing or the processing environment.

Candida parapsilosis (62%) and *Lodderomyces elongisporus* (25%) were the dominant species isolated from the nozzles (area E) which release the fruit juice into the bottles during filling. Both yeasts are common contaminants in bottling factories, but can be effectively controlled if GMPs are strictly adhered to (Davenport, 1996). *Cryptococcus saitoi* (13%) was also isolated from the nozzles. *C. parapsilosis* (33%), *L. elongisporus* (33%), *Candida sojae* (12%), *Candida oleophila* (12%) and *Cryptococcus laurentii* (17%), were isolated from the bottles and caps which are used for packaging during filling. Caps and bottles (area F) are not washed prior to filling and are stored in the roof area which is not properly insulated. The latter is likely to introduce soil and dust-related yeasts such as *L. elongisporus* and *C. laurentii* into the packaging material (Stratford and James, 2003; Slavikova et al., 2007; Cloete et al., 2010).

Wickerhamomyces anomalus was also isolated in many areas within the factory (Figure 3.4.). In a study conducted by Marvig et al. (2014), *W. anomalus* was also isolated in relatively high numbers from sugar syrups and syrup tank swab samples. *W. anomalus* is known to exhibit a great diversity with regard to its natural habitat, growth morphology, metabolism, stress tolerance and antimicrobial properties (Walker, 2011). It has been isolated from sources such as flowering plants, fruit skins, insect intestinal tracts, human tissues and faeces, wastewaters, dairy and baked food products. There are some detrimental roles of *W. anomalus* in relation to food production and storage (Deak, 2008). As a food spoilage yeast, its contamination of sugary foods and beverages (Lanciotti et al., 1998; Rojas et al., 2001) can lead to taints commonly referred to as "chemical adulteration".

Zygosaccharomyces bailii was not widely distributed in the processing equipment analysed, although it has been reported as one of the main spoilage yeasts associated with fruit juice (Figure 3.4) (Stratford et al., 2013b). This observation can be linked to the fact that it exhibits the lowest capacity of adhesion to stainless steel along with the lowest percentage of hydrophobicity (Brugnoni et al., 2007). Stainless steel is the most frequently used food contact material in the fruit juice processing industry. The two above mentioned cumulative parameters combined, leads to a significant decrease of the adhesion capacity of this strain (Brugnoni et al., 2007). Even though the presence of *Z. bailii* may be low on stainless steel, it can multiply gradually in concentrates and as little as one cell per container of diluted stock is enough to cause spoilage (Wareing and Davenport, 2000). Flavourings, fruit pulp and water can also be potential sources of contamination.

In conclusion, the diversity data revealed that this fruit juice factory environment harbours a variety of yeast species, even after cleaning and disinfection. Some of these yeasts are common contaminants of fruit juice and could potentially affect the product as spoilers. Furthermore, the presence of hygiene indicator yeast species in the factory environment suggests that the current GMPs could be improved.

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Chapter 4

Application of RFLP analysis for preliminary identification of unknown spoilage yeasts in fruit juices

4.1 Introduction

Early detection of microbial contamination is of considerable importance to the food industry and provides a means of monitoring quality control in production processes. It also enables quicker implementation of intervening measures, thus reducing the effects of potential spoilage (Loureiro, 2000; Casey and Dobson, 2004; Combina et al., 2008). The most common microbial indicator used by the food industry to evaluate the presence of foodborne yeasts is still enumeration of total yeasts and mould making use of rich culture medium (Combina et al., 2008) supplemented with antibiotics, such as chloramphenicol and oxytetracycline, to prevent bacterial growth (Mossel et al., 1995). Such non-descriptive media cannot distinguish between dangerous and innocuous yeasts, making it impossible to determine whether more than one species of yeast may be present. Furthermore, if the identity of the spoiler/contaminant is not known, it becomes extremely difficult to decide on the suitable preventative measure to be taken in cases of high levels of contamination (Loureiro, 2000).

Traditionally, yeast identification has been based on morphological and physiological traits. However, these methodologies are laborious and time consuming (Baffi et al., 2011). Traditional, labour-intensive, physiological methods have largely been replaced by molecular techniques (Kurtzman, 2006; Pincus, 2007). Each method has its own advantages and disadvantages with respect to speed, reliability, robustness, scale, cost, infrastructure requirements, training and expertise, and these will always need to be evaluated with respect to the requirements of the user (Harrison et al., 2011). There are many recent, complex and sensitive identification tools such as Next Generation

Sequencing (NGS), metagenomics, Matrix–Assisted Laser Desorption/Ionisation (MALDI) and Fourier Transform Infrared Spectroscopy (FTIR) for rapid identification of pathogens and spoilers, but for small scale fruit juice bottling factories, the need exists for a basic and fast method, at least for preliminary identification.

According to Baffi et al. (2011), reliable identification can be achieved by restriction analysis (PCR-RFLP) of non-coding ribosomal DNA regions. These regions include, but are not limited to, the internal transcribed spacers and the 5.8S region (ITS-5.8S region) of the large 26S subunit of rRNA. RFLP relies on the use of restriction enzymes to digest DNA into fragments of various lengths that may be species-specific (Kurtzman, 2015). Usually the DNA to be digested represents an amplicon of e.g. combined ITS and D1/D2 regions to ensure an adequate DNA concentration for detection on gels (Ferreira et al., 2010). Comparisons are usually based on the use of patterns that have been generated by several different restriction enzymes.

The fruit juice bottling factory where samples were obtained for this study performs in-house microbial culturing. However, the factory is not equipped with laboratory facilities to identify spoiler yeasts to species level. Moreover, species identification is a lengthy process if outsourced which hampers planning of corrective actions. Therefore, the aim of this study was to apply a PCR-RFLP approach in the preliminary identification of spoilage yeasts associated with spoiled products from a fruit juice bottling factory in Bloemfontein.

4.2 Materials and methods

4.2.1 Spoiled fruit juice samples

Eight fruit juice samples, each representing a different batch affected by spoilage (blowing), were collected, retained on ice during transportation to the laboratory and analysed without delay. Four different fruit juice flavours were sampled including, Apple Ice-Tea, Jungle Yum Cordial, Peach and Apricot and Coco Pine Dairy Blend.

4.2.2 Enumeration and isolation of yeasts

Fruit juice samples were serially diluted in sterile peptone water (Merck). The series of dilutions were plated onto RBC agar as well as Malt Extract agar (MEA) and incubated for up to 3 days at 30°C. Of the resulting colonies, approximately 10% were selected based on colony morphology and used as template in whole cell PCR (Barata et al., 2008). Isolates were also purified by repeated sub-culturing and cryopreserved in 15% glycerol at -20°C.

4.2.3 Control panel for comparison

A representative of each yeast species characterized in the diversity study (Chapter 3), as well as reference strains, were included as a control panel to serve as comparisons for the application of RFLP analysis.

4.2.4 PCR amplification and 5.8S–ITS rDNA RFLPs

Whole-cell PCR and restriction enzyme digestion were performed on the unknown isolates as well as the control panel yeasts. Yeast cells from 48 h single colonies were suspended in 50 µl PCR reaction mix containing 0.52 µM primer ITS 1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al., 1990), 0.2 µM dNTPs, 1x reaction buffer Thermopol® (New England Biolabs), and 1 U of NEB Taq Thermopol® (New England Biolabs). Amplification conditions included initial denaturation at 95°C for 3 min, followed by 30 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 1 min. A final elongation step was performed at 68°C for 7 min. Successful amplification was confirmed by agarose gel (1%) electrophoresis. PCR products were stained with ethidium bromide (Merck) and visualised under UV light. These PCR products represented the 5.8S–ITS rRNA region and varied in length from 300 – 900 bp. All amplicons (10 ul) were digested with 1 unit of *Cfo*I, *Hae*III and *Hinf*I restriction enzymes (Thermo Scientific) using FastDigest™ and Tango™ buffers (Thermo Scientific) in separate reactions (Esteve-Zarzoso et al., 1999).

Fragments were separated in a 3% agarose gel and stained and visualised as mentioned. Digital images were captured with the Molecular Imager® Gel Doc™ XR system (BioRad Laboratories Inc.). Band sizes were calculated with reference to GeneRuler™ 1 kb DNA ladder Plus and GeneRuler™ 50 bp DNA ladder (Thermo Scientific) using Quantity One® 1-D Analysis software (BioRad Laboratories Inc.). Resulting PCR-RFLPs were grouped according to profiles and compared to the diversity control panel for preliminary identification.

4.3 Results and discussion

4.3.1 Identification of unknown yeasts from spoiled fruit juices

The factory relevant to this study experiences an annual problem with 'blowing' of the fruit juice concentrates and a rapid, cost effective method is required to identify spoiler yeasts. The detection and identification of yeast species in spoiled fruit juice would be useful in providing information about the composition of yeast populations which affect the organoleptic properties of the final product (Granchi et al., 1999). Moreover, since different populations of yeasts can spoil the same batch, timely knowledge on which yeast community is present should assist with corrective actions and problem solving.

A proposed PCR-based yeast colony identification protocol, consisting of RFLP analysis of amplified 5.8S-ITS rDNA is illustrated in Figure 4.1. This protocol comprised the following steps: (1) Dilution and plating of spoiled fruit juice sample; (2) 72 h incubation; (3) direct whole cell PCR amplification of 5.8S- ITS rDNA (4) restriction analysis with enzymes (*Cfo*I, *Hae*III and *Hinf*I) and agarose gel electrophoresis; (5) digital acquisition of restriction profiles and comparison with the control panel yeasts for preliminary identification; and (6) D1/D2 domain sequencing of representative isolates for confirmation. Steps 1 and 2 can also be carried out in the factory laboratory.

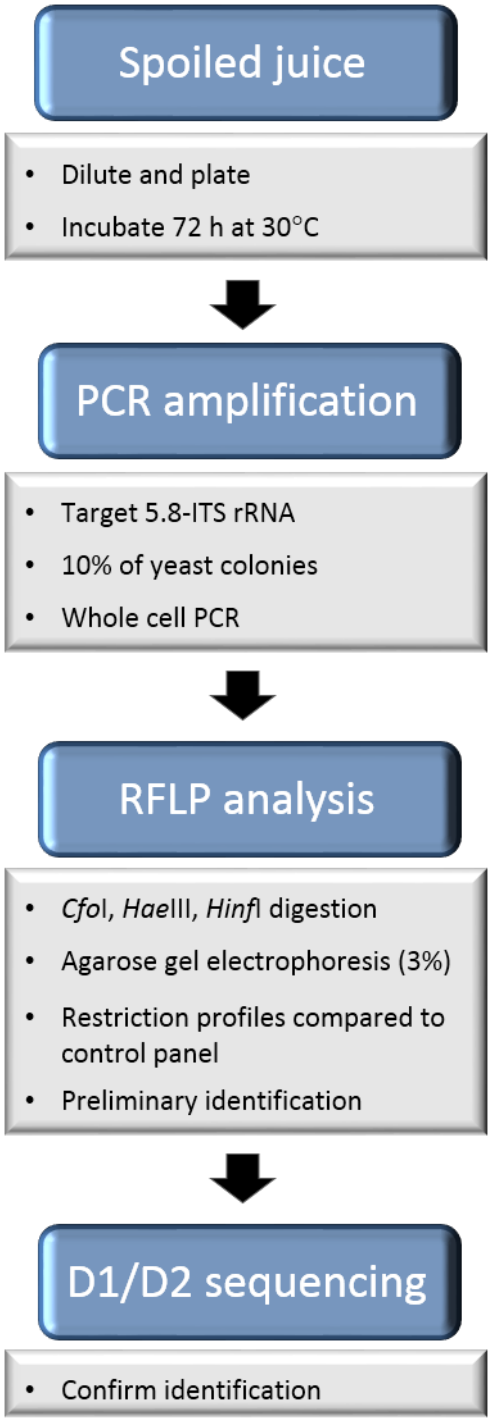

Figure 4.1 A flow diagram illustrating the 5.8-ITS rDNA RFLP based protocol for preliminary identification of yeast present in spoiled fruit juice.

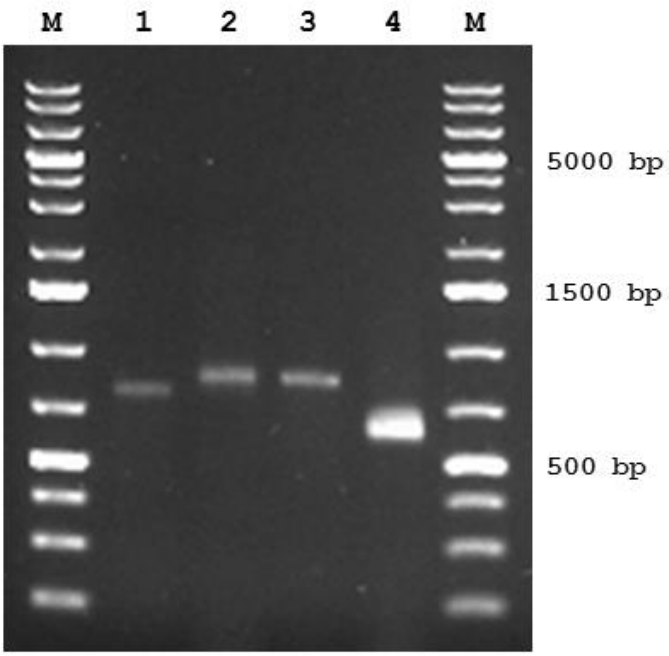

Figure 4.2 Agarose gel (1%) showing representative PCR products amplified from different yeasts isolated from each fruit juice. Lanes M- O'GeneRuler^{TM} 1 kb Plus DNA Ladder, Lane 1- Unknown yeast 1 from "Jungle Yum cordial" Lane 2- Unknown yeast 2 from "Apple Ice-Tea", Lane 3- Unknown yeast 3 from "Coco pine dairy blend" and Lane 4- Unknown yeast 4 from "Jungle Yum cordial".

Yeasts were isolated from eight spoiled fruit juices each representing a different batch affected by spoilage. The yeast populations ranged from 2.80×10^3 to 2.23×10^7 CFU/ml which was within the same range isolated by Deak and Beuchat (1993) in fruit juice concentrates. The analytical methods used by the industry to evaluate yeasts present in foods and drinks are still yeast and mould count plates, making use of a general rich medium (Combina et al., 2008). Under these conditions, it not possible to distinguish between the diverse yeasts causing spoilage and their different characteristics. This makes it difficult to determine the preventative measures that must be taken in cases of high levels of contamination (Loureiro, 2000).

Following 5.8S-ITS rDNA amplification from the resulting yeast colonies, the PCR product lengths were between 500 and 900 bp and already showed that spoilage of the different juices were likely caused by the presence of different yeast species (Figure 4.2). Even so, as was demonstrated in the Chapter 3, PCR product length alone is not enough for dependable identification. PCR products were digested with *Cfo*I, *Hae*III and *Hinf*I. Figure 4.3 depicts the digestion profiles of unknown yeasts and control strains, which include yeasts identified from the diversity study as well as all the reference strains. Unknown yeasts which were isolated from spoiled fruit juice concentrates are represented in Lanes 13, 23, 26 and 30. The restriction profiles of the unknown spoilers were compared to that of the control panel and subsequently identified. The restriction profile of Unknown yeast 1 (Figure 4.3 Lane 13) was not identical to any of the isolates from the control panel but closely resembled that of *Zygosaccharomyces bailii*. Preliminary identification was not possible for this specific profile, since it did not have a match in the control panel. The isolate was sequenced (D1/D2 domain) and identified as *Zygosaccharomyces bisporus*. The restriction profile of Unknown yeast 2 (Figure 4.3 Lane 23) was identified as *Zygosaccharomyces bailii* (Figure 4.3 lane 31). Similarly, the restriction profiles of Unknown yeast 3 (Figure 4.3 lane 26) was identical to that of *Saccharomyces cerevisiae* (Figure 4.3 lane 32) and the restriction profile of Unknown yeast 4 (Figure 4.3 lane 30) was identical to *Zygoascus hellenicus* (Figure 4.3 lane 33). The identities of Unknown yeasts 2, 3 and 4 were also verified by D1/D2 domain sequencing.

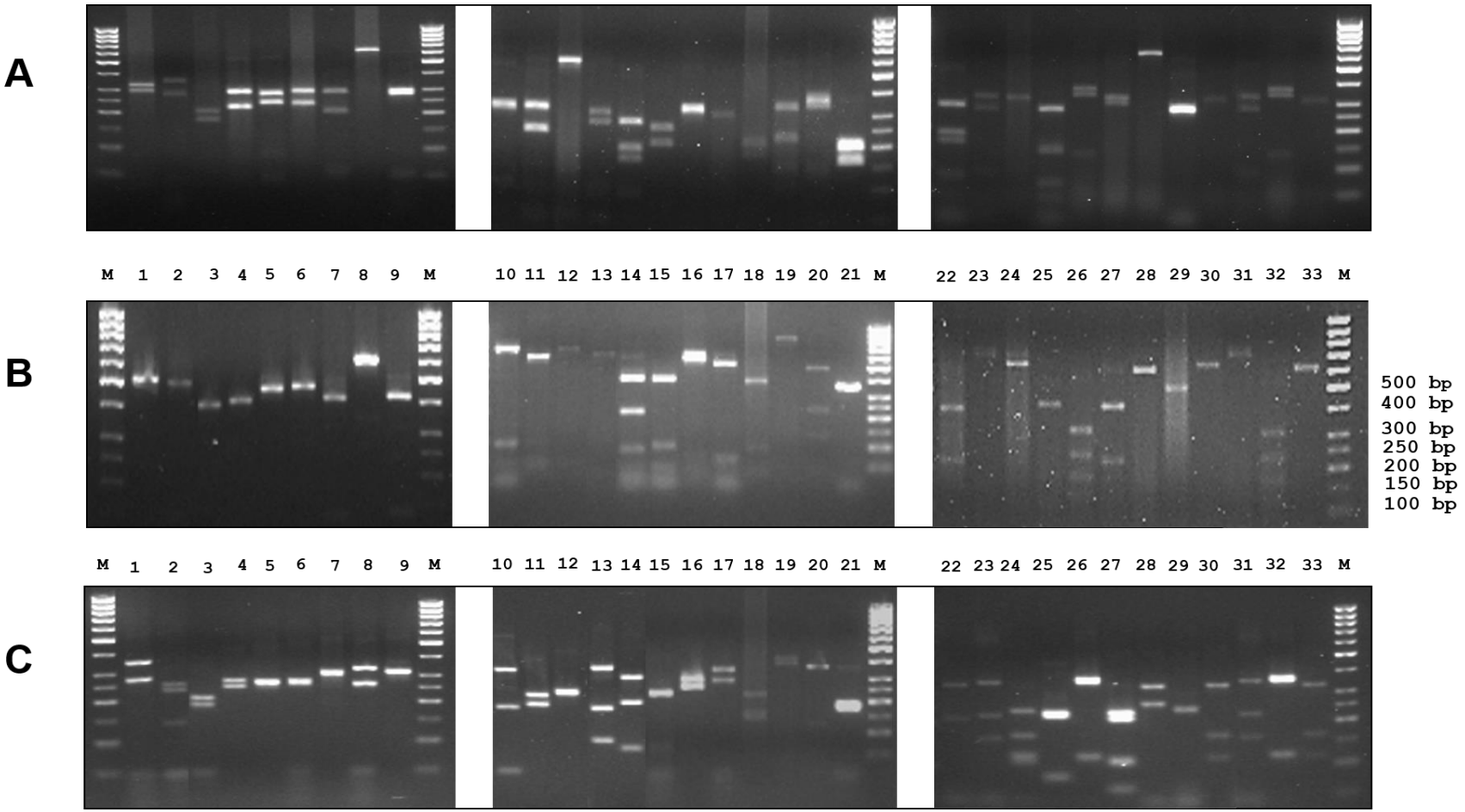

Figure 4.3 Composite RFLP panel of yeasts isolated from the factory environment (control panel), selected reference strains and Unknown yeasts isolated from spoiled fruit juice. The agarose gels (3%) show restriction profiles generated by digestion with *Cfo*I (A), *Hae*III (B) and *Hinf*I (C) enzymes. Each lane represents the following in all gel sections A, B and C: M- GeneRuler™ 50 bp DNA ladder (Thermo Scientific), 1- *Filobasidium capsuligenum*, 2- *Filobasidium uniguttulatum*, 3- *Candida intermedia*, 4- *Candida parapsilosis*, 5- *Candida tropicalis*, 6- *Candida sojae*, 7- *Candida quercitrusa*, 8- *Candida spandovensis*, 9- *Candida oleophila*, 10- *Saccharomyces ludwigii*, 11- *Lodderomyces elongisporus*, 12- *Wickerhamomyces anomalus*, 13- **Unknown yeast 1**, 14- *Zygosaccharomyces rouxii*, 15- *Cryptococcus laurentii*, 16- *Cryptococcus saitoi*, 17- *Millerozyma farinosa*, 18- *Pichia kudriavzevii*, 19- *Torulaspora delbrueckii*, 20- *Saccharomyces bayanus*, 21- *Yarrowia lipolytica*, 22- *Kluyveromyces marxianus*, 23- **Unknown yeast 2**, 24- *Hanseniaspora occidentalis*, 25- *Dekkera anomala*, 26- **Unknown yeast 3**, 27- *Rhodotorula dairenensis*, 28- *Cystobasidium slooffiae*, 29- *Trichosporon ovoides,* 30- **Unknown yeast 4**, 31- *Zygosaccharomyces bailii*, 32- *Saccharomyces cerevisiae* 33- *Zygoascus hellenicus.*

Table 4.1 Yeast species isolated from spoiled fruit juices

Fruit juice flavour	Yeast species
Apple Ice-Tea	*Zygosaccharomyces bailii*
Peach & Apricot	*Zygosaccharomyces bailii*
Jungle Yum Cordial	*Zygosaccharomyces bailii* *Zygosaccharomyces bisporus* *Zygoascus hellenicus*
Coco Pine Dairy Blend	*Zygosaccharomyces bailii* *Saccharomyces cerevisiae*

Distribution of the four different yeast species identified from spoiled fruit juices of different flavours is listed in Table 4.1. *Zygosaccharomyces bailii* was isolated from all the fruit juice flavours and was the only spoiler present in the Apple Ice-Tea and Peach & Apricot flavours. *Zygoascus hellenicus* was only isolated from the Jungle Yum Cordial and *Saccharomyces cerevisiae* was only isolated from Coco Pine Dairy Blend.

The 5.8S-ITS region RFLP approach to identify yeast compared to control panel data proved useful for preliminary identification of unknown spoiler yeasts in fruit juices. The presence of *Zygosaccharomyces bailii*, *Saccharomyces cerevisiae* and *Zygoascus hellenicus* were easily confirmed and a fourth species that did not form part of the control panel (*Zygosaccharomyces bisporus*) was immediately noted. Furthermore, the RFLP approach was also able to indicate the presence of more than one yeast species in spoiled fruit juice and provided guidance toward deciding how many representative isolates to sequence for confirmation.

All yeasts isolated from the spoiled fruit juices fall under Group 1 yeasts (Davenport, 1996). These are described as spoilage organisms that adapted to growth in fruit juices and are able to cause spoilage from very low cell numbers (as few as one cell per container). The characteristics of Group 1 yeasts are osmotolerance, aggressive fermentation, resistance to preservatives (particularly weak organic acids) and a

requirement for vitamins. It is the high sugar concentrations in fruit juice concentrates that favour the growth of yeasts with a higher fermentative activity (Barata et al., 2012).

The genus *Zygosaccharomyces* including both *Z. bisporus* and *Z. bailii* are some of the species that have evolved the ability to grow under difficult environmental conditions (Merico et al., 2003). Low pH, high sugar concentration and low water activities provided by the fruit juices, encourage proliferation of these species (Fleet, 1992). Another feature which may contribute to the spoilage capacity of yeasts belonging to this genus is their ability to vigorously ferment hexose sugars, such as glucose and fructose (Thomas and Davenport, 1985; Pitt and Hocking, 1997). High fermentation can contribute to spoilage by causing swelling in packaging which was observed in the plastic juice bottles in this study.

Zygosaccharomyces bailii is a commonly encountered spoilage yeast and this species is responsible for considerable economic losses in the beverage industry (Thomas and Davenport, 1985; Loureiro, 1994; Rojo et al., 2014). *Z. bailii* was detected in all the spoiled fruit juice flavours studied and is not unexpected given its characteristic for extreme resistance to preservatives and ability to grow in excess of legally permitted concentrations of preservatives (Harrison et al., 2011; Stratford et al., 2013b). The low permeability of *Z. bailii* to weak acid preservatives at low pH values and its ability to metabolize acid compounds, even in the presence of glucose, are some of the physiological traits associated with its high tolerance to acidic environments (Sousa et al., 1996; Fernandes, 1997; Sousa, 1998). The factory from which the spoiled fruit juices were obtained for this study uses sodium benzoate and sodium metabisulphite preservatives, which are classified under weak acid preservatives, and are thus easily resisted by members of the genus *Zygosaccharomyces*. The frequency of *Zygosaccharomyces bisporus* isolation from foods is much lower than that of *Z. bailii*, but it has a similar ability to cause food spoilage and it is also preservative-resistant (Pitt and Hocking, 1999; Barata et al., 2008).

Zygoascus hellenicus also has a high fermentative ability and the available literature extensively describes it as being associated with grape berries or must in the winery environment (Barata et al., 2008; Barata et al., 2012; Tristezza et al., 2009; Simões and

Gomes, 2015). The Jungle Yum fruit juice which is the only fruit juice type contaminated by *Z. hellenicus* consists of strawberry, cranberry and raspberry pulp. It is possible that this species originated from the pulp since it has been associated with contamination of berries. It has also been described as a contaminant often associated with damaged grapes (Barata et al., 2008) and some studies indicated that it has been isolated from fruit juices (Nyanga et al., 2007; Maciel et al., 2013).

It was not unusual to isolate *Saccharomyces cerevisiae* since it is also a fermentative yeast which has been associated with microbial decomposition of fruit juices (Zook et al., 1999; Turtoi, 2014). *Saccharomyces cerevisiae* is considered to be a predominant spoilage species in concentrates, juices and fruit beverages (Gardini and Guerzoni 1986; Deak and Beuchat, 1993), and in that respect is considered to be the source of most problems associated with processed fruits (Maimer and Busse, 1992). It has been isolated from fruit juices and causes ethanoic spoilage, carbonation, production of hydrogen sulphide and other off- odours (Zook et al., 1999). This yeast can grow in a range of conditions and it is characterized for its optimal growth in high sugar content media. *Saccharomyces cerevisiae* has also been isolated from a variety of dairy products especially those containing sugar and fruit (Mayoral, 2005). Not surprisingly, the Coco-pine dairy blend which contains milk powder was contaminated by *S. cerevisiae*. In addition, *S cerevisiae* has a level of tolerance to benzoic acid and is able to degrade sorbic acid (Pitt, 1974; Stratford et al., 2007).

In conclusion, the method of applying RFLP analysis of the 5.8S-ITS region was useful for preliminary identification of spoilage yeasts in fruit juice. The main spoilage yeasts were identified to species level and with this information, the contamination sources during fruit juice processing can be determined. This method was also effective in detecting mixed cultures in a spoiled product. These spoilage yeasts can now be specifically targeted when developing contamination control measures.

4.3.2 Origin of contamination

From the yeast diversity data obtained in Chapter 3 it was noted that *Zygosaccharomyces bailii* and *Zygoascus hellenicus* were both isolated from the air in the refrigerator where the fruit pulp is stored (Figure 4.4), which may suggest the refrigerator as the likely source of contamination. *Z. bailii* is able to grow at refrigerator temperatures, and therefore capable of surviving in this particular setting (Steels et al., 1999). *Wickerhamomyces anomalus* and *Rhodotorula slooffiae* were also isolated from the fridge, but was not present in the final product (Figure 4.4). Although *W. anomalus* is able to grow over a broad pH range and at high osmotic pressure, its growth can be inhibited by benzoic acids and its derivatives (Passoth et al., 2006; Koczoń, 2009). Sodium benzoate is one of the preservatives used in the fruit juices and could have prevented the growth of *W. anomalus*. *Z. bailii* is preservative resistant and would therefore be likely to thrive in the products. There is very limited information about *Rhodotorula slooffiae* isolation from food products and its sensitivity to preservatives.

Zygoascus hellenicus was also isolated in the blending tanks while *Zygosaccharomyces bailii* was isolated in the powder blenders (Figure 4.4). *Saccharomyces cerevisiae* and *Zygosaccharomyces bisporus* were not isolated in the factory environment during the diversity study which shows that spoilage yeasts do not necessarily originate from the factory environment. Although it is unlikely that these micro-organisms were missed during sampling it cannot be excluded as a possibility. It is, however, more reasonable to assume that they originate from an external source; presumably the concentrated pulps.

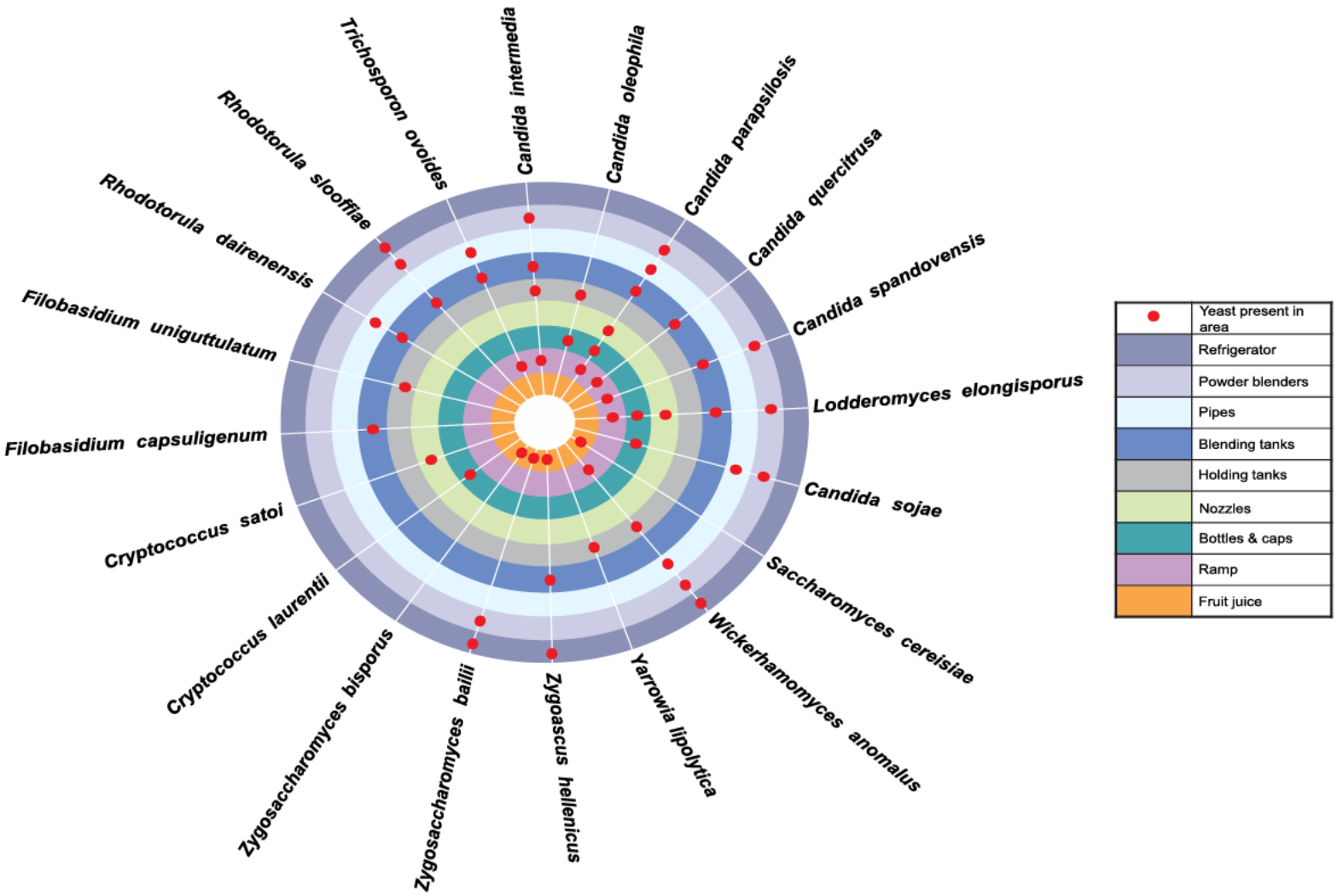

Figure 4.4 Data wheel showing the yeast distribution in the fruit juice bottling factory and the spoiled products. Yeasts isolated from the refrigerator (area A), powder blenders, pipes, blending tanks (area C), holding tanks, nozzles (area E), bottles, caps (area F), ramp (area D) and fruit juice.

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Chapter 5
Concluding remarks

5.1 Diversity and source of contamination

A total of 201 yeasts belonging to ten different genera (*Candida*, *Lodderomyces*, *Wickerhamomyces*, *Yarrowia*, *Zygosaccharomyces*, *Zygoascus*, *Cryptococcus*, *Filobasidium*, *Rhodotorula/Cystobasidium and Trichosporon*) were isolated and identified from the production environment and processing equipment of a fruit juice bottling facility. Ascomycetous yeasts were dominating and included *Candida intermedia, Candida oleophila, Candida parapsilosis, Candida quercitrusa, Candida spandovensis, Candida sojae, Lodderomyces elongisporus, Wickerhamomyces anomalus, Yarrowia lipolytica, Zygoascus hellenicus* and *Zygosaccharomyces bailii*. Basidiomycetous yeasts were represented by *Cryptococcus laurentii, Cryptococcus saitoi, Filobasidium capsuligenum, Filobasidium uniguttulatum, Rhodotorula dairenensis, Cystobasidium slooffiae/Rhodotorula slooffiae* and *Trichosporon ovoides*.

Factories producing fruit products can be at risk of yeast contamination due to its high sugar, low water activity and low pH (Ridawati et al., 2010). In such factories, care must be taken to ensure that equipment such as storage chillers, blending tanks, pipes, powder blenders, fillers and other product contact surfaces are adequately sanitised. The overall yeast distribution shows that *Candida parapsilosis* and *Lodderomyces elongisporus* were widely distributed in the factory. *C parapsilosis* is not only a yeast that contaminates fruit juices, but also an opportunistic human pathogen which can cause infections in immuno-compromised patients (Maciel et al., 2013). Therefore, proper food handler hygiene practices should be established. Although not isolated from the fruit juices, both these yeasts may potentially contaminate the product (Chang et al., 2012).

Yeasts from ten different genera were isolated from the factory environment but only four species from three genera caused spoilage of the fruit juices. These yeasts were identified as *Zygosaccharomyces bailii, Zygosaccharomyces bisporus, Zygoascus*

hellenicus and *Saccharomyces cerevisiae.* Yeasts which were causing spoilage of the fruit juices were not isolated on the majority of the equipment from the factory environment. However, it only requires very low levels of particular spoilage yeasts to create major spoilage problems in these products. The yeast counts ranged from 2.80×10^3 to 2.23×10^7 CFU/ml in the spoiled fruit juices. *Zygosaccharomyces bailii* was isolated from all the spoiled fruit juice types making this particular yeast the factory's main problem.

Hygiene indicators such as *Trichosporon* spp. and *Candida* spp. were detected and all yeasts from the processing equipment were isolated after Cleaning in place (CIP). This suggests that disinfectant efficacy and yeast tolerance or resistance requires further investigation.

5.2 Preliminary identification using RFLP

Application of RFLP analysis of the 5.8S-ITS region proved to be a useful tool in detecting genetic variability among yeast species, which is valuable for taxonomic identification to species level. This study established a RFLP profile database for yeast species isolated from this particular fruit juice factory. All the yeast species isolated from the factory environment serves as a control panel with which unknown spoilers are compared. Once the database of species has been expanded; rapid, reliable and cost effective identification of yeast species from fruit juice will be possible. This method not only enables quick identification, but also provides insight into whether the spoiler can be attributed to the factory environment or an external source.

In general, the results demonstrate good reliability of the 5.8S-ITS analysis as a routine technique for identification of fruit juice yeast isolates. This method allows preliminary identification within 72h which can be less if single colonies are provided from the factory's routine testing. Restriction digest profiles for *Zygosaccharomyces bisporus* were not present in the control panel database and could not be identified using this technique. In this case, species assignment was based on partial sequence of the 26S rDNA gene. Isolation of a yeast species from fruit juice which did not have a match in the

existing database highlighted the necessity of updating the control panel database on a regular basis.

The preliminary identification method applied in this study, also provides useful information on spoiler diversity and guides on the number of colonies to sequence for confirmation. In so doing, sequencing costs may be reduced. Furthermore, testing the concept of using an RFLP profile database was aided by the inclusion of reference yeast strains in addition to isolates. The construction of a database including reference and local strains proved to be very important to assure speed of identification.

5.3 Recommendations

Osmotolerant yeasts such as *Zygosaccharomyces bailii* are ideally suited to the environmental niches in these production environments, hence the need for good hygiene in these areas. In view of their resistance to preservatives and low tolerance to cleaning agents, *Z. bailii* and *Zygosaccharomyces bisporus* may be better prevented by the use of biocidal cleaning agents, rather than treating the food with preservatives (Martorell et al., 2007; Hayes et al., 2012). This may be effective since Martorell et al. (2007) found that *Z. bailii* isolates were not exceptionally resistant to biocides such as peracetic acid or hypochlorite.

Dimethyldicarbonate (DMDC) is an antimicrobial agent that has recently been approved for the control of spoilage yeasts in wines (Martorell at al., 2007; OIV, 2013). This chemical compound may be used as a preservative in fruit juice. In a synthetic medium, the inhibitory activity of DMDC was yeast species and dose dependent, where 0.112 mM of DMDC were necessary to reach the complete inhibition of *Z. bailii* (Delfini et al., 2002). Moreover, a combination of DMDC (0.07 mM) and natamycin (0.015 mM) is also recommended. Vanillin, a naturally derived inhibiting additive can also be used to inhibit the growth of yeasts in fruit juice. Cerrutti and Alzamora (1996) showed that growth of *Saccharomyces cerevisiae, Zygosaccharomyces rouxii, Z. bailii* and *Debaryomyces hansenii* was inhibited in culture media and apple purée containing 13 mM of vanillin. Additionally, vanillin was inhibitory to *Z. rouxii* and other yeasts at concentrations of

20 mM, but lower concentrations were also effective when combined with other harsher conditions such as lower temperatures and low pH (Fitzgerald et al., 2003).

The present study has demonstrated that the processing equipment of fruit juice factories potentially contains a wide diversity of yeast species both during production and after cleaning and disinfection. The contamination of equipment by yeasts is usually attributed to poor hygienic practices; however, the resistance of yeasts to commercial sanitizers and cleaning compounds has been reported (Laubcher and Viljoen, 1999). Hygiene indicators such as *Trichosporon* spp. and *Candida* spp. were detected from all the processing equipment after CIP. This suggests that disinfectant efficacy and yeast tolerance or resistance requires further investigation.

A variety of yeasts were isolated from the packaging material which confirms the importance of proper hygiene, sanitation and good household practices, not only of the product and production equipment, but also of any containers and packing materials. These too can be a source of spoilage yeasts. The proper storage of such containers and closures before use is also important because yeast numbers may increase substantially during storage. Insulating the second floor area from which the caps and bottles are dispensed should assist with limiting yeast contamination of packaging materials.

5.4 Future Research

For successful application of the preliminary identification method, ongoing monitoring of yeast diversity is recommended. This will enable the control panel database to be continuously updated. Furthermore, evaluating the efficacy of the disinfectants, sanitizers and preservatives which are currently used in the factory, on the yeasts isolated from this study is the logical next step. Investigating food handlers and other factory personnel as sources of yeast-related contamination, linked with particular behaviours, should also be of considerable benefit to the industry.

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