BACTERIAL CONTAMINATION OF COMMERCIAL YEAST

Susannah Sara O'Brien

A dissertation submitted to the Faculty of Science, University of the Witwatersrand, in fulfillment of the requirements for the degree of Masters of Science.

Gauteng, 2004

DECLARATION

I declare that this is my own, unaided work. It is being submitted for the degree of Master of Science in the University of the Witwatersrand, Gauteng. It has not been submitted before for any degree or examination in any other University.

_____

Susannah Sara O'Brien

_____ day of _____, 2005.

TABLE OF CONTENTS

PREFACE

Some aspects of the work conducted for this dissertation have been or will be presented as papers or posters elsewhere:

CHAPTER 2

O'Brien, S. S., Tessendorf, B. A., Brodie, M., Lindsay, D., von Holy, A. (2003). Bacterial contamination of commercial yeast. International Association for Food Protection (IAFP), 90th Annual Meeting, New Orleans, Louisiana.

O'Brien, S. S., Tessendorf, B. A., Brodie, M., Lindsay, D., von Holy, A. (2003). Survey of a commercial yeast manufacturing process for bacterial contamination. South African Association for food Science and Technology (SAAFoST), 17th Annual Meeting, CSIR Conference Centre, Pretoria.

O'Brien, S. S., Lindsay, D., von Holy, A. (2004). The presence of *Enterococcus*, coliforms and *E. coli* in a commercial yeast manufacturing process. International Journal of Food Microbiology 94, 23 – 31.

O'Brien, S. S., Lindsay, D., von Holy, A. (2004). Survey of a commercial yeast manufacturing process for sources of spoilage and potentially pathogenic bacteria. International Association for Food Protection (IAFP), 91st Annual Meeting, Phoenix, Arizona, U.S.A.

CHAPTER 3

O'Brien, S. S., Lindsay, D., von Holy, A. (2004). Bacterial populations associated with extended storage of commercially manufactured yeast. International Association for Food Protection (IAFP), 91st Annual Meeting, Phoenix, Arizona, U.S.A.

CHAPTER 4

O'Brien, S. S., Lindsay, D., von Holy, A. (2004). Biofilm formation on yeast processing equipment surfaces as studied by scanning electron microscopy. Proceedings of the Electron Microscopy Society of Southern Africa 34, 39.

O'Brien, S. S., Lindsay, D., von Holy, A. (submitted). *In situ* biofilms associated with Baker's yeast processing equipment. International Association for Food Protection (IAFP), 92nd Annual Meeting, Baltimore, Maryland, U.S.A.

ABSTRACT

The bacterial contamination profile of a typical commercial yeast factory was assessed by three replicate microbiological surveys. In order to detect low-level contamination in samples, this study made use of a preliminary incubation technique (24h at 37°C), which boosted bacterial counts for the identification of sources of contamination. Numbers of bacteria were quantified by standard pour- and spread-plate techniques and various selective media. Raw materials were negligible in contributing to the bacterial contamination of commercial yeast, with the exception of soda ash, used to control the pH of fermentations, which contained 2 log CFU/ ml *Enterococcus* and aerobic bacteria. It was found that the scale up of seed yeast biomass was the primary site for contamination with *Enterococcus*, which progressively increased in number as the product passed down the production line. Coliforms were present at low levels, with significant increases ($P < 0.05$) observed during the storage of yeast cream; extrusion of compressed yeast; and packaging of dry yeast. The environment surrounding the compressed yeast production line was identified as a potential source of airborne contamination. Although *Salmonella* spp. and *S. aureus* were not detected, *L. monocytogenes* was isolated from compressed and dry yeast products. In addition, *Bacillus* spp. commonly associated with the rope-spoilage of bread, were isolated from 67% of all dry yeast product samples. Shelf-life investigations, showed that cream and compressed yeast samples were spoiled with lengthened storage periods, and especially at higher temperatures (>10°C), whilst vacuum-packaged dry yeast remained bacteriologically stable. During shelf-life studies, isolates from spoiled cream and compressed yeast samples were predominantly *Lactobacillus* (up to 78%), while populations of *Enterococcaceae* predominated in vacuum-packaged dry yeast samples (up to 68%). The use of stainless steel surfaces, attached to processing equipment used in the manufacturing of Baker's compressed yeast, in conjunction with SEM illustrated the accumulation of yeast and bacterial cells with early stages of biofilm formation, with time. Where populations of Gram-positive members of the lactic acid bacteria family, *Lactobacillus* and *Enterococcaceae*, were isolated in the highest proportion from processing equipment surfaces used in the manufacturing of Baker's compressed yeast (81-100%).

LIST OF FIGURES

LIST OF TABLES

Dedicated, with love, to my parents, **Eileen and Stephen O'Brien**, my sisters, **Jennifer Gouveia** and **Catherine Davis**, and my fiancée, **Gerry Roelofsz**.

ACKNOWLEDGEMENTS

I would hereby like to extend my sincere thanks to the following people and institutions, without whom this study would have been impossible:

To my supervisor, Professor Alex von Holy, for his advice, enthusiasm and support throughout the course of the study.

To my advisor, Doctor Denise Lindsay, for her greatly appreciated experimental advice and proofing of this document.

Special thanks to Mrs. Michelle Brodie, Mr. Mark Perryman, Mr. Gert Steenkamp and Mr. Brett Tessendorf, for their logistical assistance, support and personal communication throughout this study.

To Anchor Yeast (Pty) Ltd. and the University of the Witwatersrand Postgraduate Merit Award (PGMA) for the financial support which made this study possible.

The financial assistance of the National Research Foundation (NRF) towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at, are those of the author and are not necessarily to be attributed to the NRF.

To my family and friends for all their encouragement throughout my studies.

Lastly, I would like to thank my fiancée, Gerry, for his endless devotion to helping me achieve. Thank you for your patience and immeasurable love.

CHAPTER ONE

INTRODUCTION

INTRODUCTION TO COMMERCIAL YEAST

Yeast are unicellular, microscopic organisms belonging to the kingdom of the Fungi. At present there are 60 genera of yeast subdivided into 500 different species according to where they are found, their cellular morphology, physiological characteristics and how they reproduce (Hough, 1985; Reed and Nagodawithana, 1991). Reproduction of yeast is achieved by budding; each cell forms a bud on the surface of the cell wall which gradually enlarges into a daughter cell containing identical chromosomes to the parent cell. Under ideal conditions the formation of a new yeast cell takes place every four hours. Through a process of fermentation, yeast cells metabolize carbohydrates into carbon dioxide and alcohol. Very few species of yeast are used commercially, but some species have proven to be beneficial to man, one of which is *Saccharomyces* (*S.*) *cerevisiae*. *S. cerevisiae* is the most important variety of all yeasts and is used in a range of industrial operations such as in the manufacturing of bread, beer, wine, and distilled beverages (Walter, 1953; Legan and Voysey, 1991; Reed and Nagodawithana, 1991).

THE FUNCTION OF YEAST IN BREAD MAKING

The use of yeast to leaven dough for bread making is one of the oldest examples of man's employment of microorganisms (Enfors, 2001). The history of the application of yeast in bread making covers a period of 6000 years, beginning with the Ancient Egyptians who discovered that the “contamination” of unleavened dough with yeasts and lactic acid bacteria (LAB) present in the atmosphere resulted in leavened bread (van Dam, 1986; Reed and Nagodawithana, 1991). The manufacture of Baker’s yeast, composed of living cells of aerobically grown *S. cerevisiae*, has become a sophisticated process and a highly-specialized industry (Enfors, 2001). The function of yeast cannot be successfully replaced by any other ingredient, thus it is vital to the baking trade. Baker’s yeast is propagated under controlled conditions, in order to ensure consistent end product quality (Pattison and von Holy, 2001). Therefore, the yeast strain used must display constant characteristics, such as the capability of fast growth, good leavening activity and genetic stability (Oura *et al*., 1982). Baker’s yeast is available in the form of yeast cakes, yeast cream and instant active dry Baker’s yeast (Doyle *et al*., 1997).

Bread, in various forms, has been a staple in the diets of many population groups for several centuries (Doyle *et al*., 1997). Modern commercial bread making follows the Chorleywood bread process and features a number of manufacturing steps: preparation of raw materials, mixing, dividing, proving, moulding, and baking at 200 - 300°C (Doyle *et al*., 1997; Pattison, 2000). The leavening of dough requires the addition of 1 to 6% yeast based on the weight of flour (Doyle *et al*., 1997). Alpha- and beta-amylases present in the moistened dough release sugars, maltose and sucrose from starch (Prescott *et al.*, 1996). The continuous production of carbon dioxide through the fermentation of sugars by the yeast cells increases the volume of the dough (Enfors, 2001; Pattison and von Holy, 2001). The carbon dioxide, constituting 40% of the increase in volume of bread, is entrapped within the gluten structure of the dough (Doyle *et al*., 1997). Baking stabilizes the dough structure producing leavened bread of a characteristic light and spongy texture, and once the internal temperature of the loaf reaches ± 55°C or higher, the yeast is killed rapidly. The small amount of alcohol produced by fermentation evaporates during baking. The second function of yeast in fermented dough is in the development of the characteristic aroma, flavour and colour of baked products (Oura *et al*., 1982; Lues, 1992). Other sources of aerating dough can be used, such as carbon dioxide injection or baking powder with mechanical mixing, however, one would not achieve the deliciously characteristic aroma and flavour achieved with Baker's yeast.

THE FUNCTION OF YEAST IN BREWING AND WINE MAKING

Alcoholic beverages are produced from a range of raw materials but especially from cereals and fruits (Ward, 1992). The strains of *S. cerevisiae* used for beer and wine production differ from Baker's yeast mainly in their nutrient requirements and utilization (Enfors, 2001). Yeast play an important role in the brewing and wine making process, not only by converting fermentable sugars to ethanol and carbon dioxide, but also by contributing to the overall flavour of the beer and wine (Reed and Nagodawithana, 1991; Ward, 1992). *S. cerevisiae* is used in beer and wine fermentations and final ethanol concentrations of 12 – 14% can be attained (Wainwright, 1992; Ward, 1992).

In brewing, malted cereals are used as the primary source of fermentable sugars. After soaking and germination at 15 to 21°C, the malt is kilned, mashed, filtered, clarified, heated and

inoculated with yeast (7 log cells/ ml) (Wainwright, 1992; Ward, 1992; Prescott *et al.*, 1996). In the production of ale, top-fermenting yeasts ferment at 15 to 22°C, rise to the surface at the end of fermentation and are skimmed off. In the production of lager, bottom-fermenting yeasts which ferment at 8 to 15°C, sink to the bottom at the end of fermentation (Wainwright, 1992; Ward, 1992). During fermentation, fermentable sugars constituting 70 to 80% of the wort carbohydrates are converted to ethanol (Ward, 1992). The freshly-fermented liquor matures during a secondary fermentation (2 to 6°C for up to 4 weeks) by the residual yeast, after which additives for haze clarification, flavour, aroma, colour, foam and microbiological stability are incorporated before bottling and packaging (Ward, 1992; Prescott *et al.*, 1996).

In winemaking, grapes are mechanically crushed, and the resulting liquid 'must' is separated before sterilization, inoculation and fermentation with yeast. In red wine fermentations, temperatures of 22 - 30°C are used, which increases the extraction of colour from the skins of grapes and the production of a rich aroma, whereas white wines are fermented at lower temperatures for 3 to 5 days producing fruity wines whilst reducing the risk of bacterial contamination (Ward, 1992; Prescott *et al.*, 1996). Following fermentation, excess yeast is removed during racking and the wine is transferred to bottles or casks to mature. During aging, the wine clears and the flavour develops, after which the wine is bottled (Prescott *et al.*, 1996). Many processing variations can be used to protect the wine from microbial spoilage, for example the addition of sulphur dioxide (Prescott *et al.*, 1996).

In the late 1960's commercially manufactured dry yeast for the fermentative production of alcoholic beverages was introduced (Reed and Nagodawithana, 1991). The methods used for large-scale production of Brewer's yeast closely resemble those used for aerobic, fed-batch production of Baker's yeast. Therefore, the process described below serves as a model system for the production of both Baker's yeast (cream and compressed yeast) and Brewer's yeast (dry yeast).

THE COMMERCIAL YEAST MANUFACTURING PROCESS

The history of the commercial manufacture of yeast dates back to the middle of the nineteenth century, when yeast for baking was obtained from distillers and breweries in the form of

"fermentation foam" (Enfors, 2001). In 1846, the Vienna Process was introduced for the largely anaerobic production of dedicated yeast starters (*S. cerevisiae*) for bread making (Oura *et al*., 1982; van Dam, 1986). Based on Louis Pasteur's finding that aeration inhibits ethanol production and promotes yeast growth, the Air Process was introduced in Denmark in 1877 (Oura *et al*., 1982; Enfors, 2001). The Zulaufverfahren Process (fed-batch technique) for the production of Baker's yeast by intermittent feeding of sugar, was invented in 1917 and is still followed today (Oura *et al*., 1982; van Dam, 1986; Enfors, 2001). The flow scheme of a typical commercial yeast plant is illustrated in Figure 1.1. The main steps are: successive anaerobic fermentation (seed yeast production), aerobic fermentation (commercial yeast production), washing and separation, filtration and extrusion, compression or drying, and packaging.

The principle raw materials used in the commercial production of yeast are the pure yeast culture (*S. cerevisiae*) and molasses. Molasses is a by-product of the sugar industry containing 45 to 55 weight percent fermentable sugars in the forms of sucrose, glucose and fructose (van Dam, 1986; Reed and Nagodawithana, 1991). Before entry into the yeast manufacturing process, raw molasses is supplemented with a synthetic sugar source (a sugar supplement from maize containing 67 w/ % glucose), clarified to remove any sludge, diluted with water and sterilized at ± 127°C for 30 minutes. The resulting molasses mixture, hereafter referred to as "sterile molasses" is held in holding tanks at ± 65°C, in an attempt to prevent microbial contamination until it is needed for the fermentation processes.

The commercial yeast process features a number of manufacturing steps starting with a small inoculum of pure culture of *S. cerevisiae* which is grown up in a series of "seed yeast" fermentations of increasing size, until the amount of yeast biomass required to start the commercial fermentation is reached (van Dam, 1986; Enfors, 2001). Table 1.1 shows a typical sequence of seed and commercial fermentation stages. The seed yeast manufacturing process starts in the laboratory, where pressure vessels are inoculated with a pure culture of *S. cerevisiae* under conditions of complete sterility and limited aeration (Tessendorf, 1991). Pressure vessels are designed to maintain aseptic conditions and the contents is sterilized at 121°C (1 bar) for 15 – 30 min (Ward, 1992) which reduces the risk of microbial contamination during the early stages of seed yeast production (Tessendorf, 1991).

The yeast culture is then used to inoculate the first of a series of increasingly sized batch fermentations [Culture Vessels 1 and 2 (CV1, CV2)] and fed-batch fermentations [Culture Vessel 3 (CV3) and Mother Fermentation (MF)] of successively increasing size (Table 1.1). The last stage (MF) is carried out in fermenters as large as those used for commercial fermentation (> $100m^3$ net volume) under aerobic conditions with incremental feeding of sterile molasses, so as to minimize anaerobic or aerobic fermentation to ethanol (Reed and Nagodawithana, 1991; Tessendorf, 1991). After washing and separation of the MF by centrifugation, the seed cream is acid-treated and used to start the commercial fermentation. The addition of acid lowers the pH of the seed yeast thus preventing and/ or reducing bacterial contamination.

The commercial fermentation of yeast is an aerobic fed-batch process which is performed in fermenters (> $100m^3$ net volume) on a medium of sterile molasses, nitrogen, phosphorus, vitamins and defoamer (Enfors, 2001). Molasses not only supplies fermentable sugars as the major source of carbon and energy, but also growth factors including vitamins and minerals (van Dam, 1986; Reed and Nagodawithana, 1991; Tessendorf, 1991; Enfors, 2001). Molasses is generally deficient in nitrogen and phosphorous with respect to yeast nutritional requirements (Ward, 1992) and needs to be supplemented with additional nitrogen (ammonium salts) and phosphates as well as additional minerals (Calcium, Magnesium), vitamins (biotin and thiamine) and trace elements (Reed and Nagodawithana, 1991).

The main function of a fermenter is to provide a closed and controlled environment, which allows for the efficient growth of cells and product formation (Ward, 1992). In the commercial production of yeast, all fermenters are operated under aerobic conditions. Anaerobic conditions result in fermentable sugar consumption and the formation of ethanol and carbon dioxide, resulting in low yeast yields (van Dam, 1986; Reed and Nagodawithana, 1991). In addition, the initial rate of sugar feed must be low to avoid the accumulation of sugar and excessive ethanol formation (Enfors, 2001). In the production of commercial yeast the process is controlled in such a way that any ethanol produced is later consumed by the yeast cells as a carbon and energy source (Enfors, 2001). The initial low pH of the seed yeast used to inoculate commercial fermentations is increased during commercial fermentation to pH 5 by the addition of soda ash, with a temperature profile of 25°C to 35°C.

At the end of commercial fermentation, the yeast forms a suspension of cells in a dark brown liquid (fermentation liquor) containing the residues of the fermented sterile molasses. The yeast cells are separated from the fermentation liquor by centrifugation and washing, resulting in concentrated yeast cream (Walter, 1953; van Dam, 1986; Enfors, 2001). The yeast cream is stored in large cooling tanks at 2-5°C in order to prevent further metabolism and growth of the yeast, as well as providing protection from adverse environmental conditions and microbial contamination. During the storage of the yeast cream, the yeast is tested for standard quality parameters including gassing power, percentage dry matter, osmotolerance, storage stability, colour, smell and microbial contamination (Enfors, 2001). Once all of these parameters are evaluated and corrected if necessary, a large portion of the yeast cream in storage is delivered as cream yeast (80% moisture) in sterilized, insulated tankers directly to the customer. As the cream yeast does not pass through filtration, mixing and extrusion the yeast suffers less damage resulting in increased quality and a reduction in microbiological contamination.

Yeast cream can be further processed into compressed yeast by filtration. The yeast cream storage tank holds the separated yeast cream and acts as a reservoir supplying the filter press (Walter, 1953). The concentrated yeast cream is filter pressed into yeast cake (70% moisture). The fresh yeast cake from the filters is conveyered to hoppers and blended with small amounts of water and emulsifier to facilitate extrusion in the form of 5kg semi-solid blocks (Ward, 1992). In addition to assisting in extrusion, the emulsifier can be added to adjust the moisture content of compressed yeast whilst giving the yeast a white, creamy appearance and inhibiting water spotting of the yeast cakes. The blocks of compressed yeast are weighed, wrapped in wax paper and refrigerated at 1 - 2°C. At these temperatures a shelf-life of 3 to 4 weeks is possible with only a slight decrease in leavening capacity (van Dam, 1986).

Further processing of the yeast cream into dry yeast involves the removal of excess water from the yeast cells by filtering and drying. The yeast cream is filtered into yeast cake (60% moisture). The yeast cake is blended with small amounts of emulsifier and extruded through perforated plates in the form of a ‘yeast beard’. The yeast beard is then subjected to further drying at ± 50°C to produce dry yeast granules (4% moisture) (Reed and Nagodawithana, 1991; Enfors, 2001). After drying, the dried yeast is transferred to a storage tank and flushed with inert gas, such as nitrogen, as a means of preservation. The dry yeast is stored in either silos or bulk bags prior to

flushing with nitrogen gas. The dry yeast is then packaged in 10 g sachets or 500 g bricks in airtight vacuum-sealed foil packages and warehoused. Due to the low moisture content and reduced available oxygen for the growth of contaminants, the dry yeast can be stored without substantial loss of activity, extending the shelf-life to one year from packaging (Reed and Nagodawithana, 1991). Rehydration at 35 to 42°C is required before use in bakery products (Doyle *et al*., 1997).

CLEANING AND SANITATION OF THE COMMERCIAL YEAST PROCESSING PLANT

Since commercial yeast is not propagated under pure culture conditions in commercial fermentation stages, it contains various microbial contaminants (Tessendorf, 1991). In addition, the conditions in which yeast is grown are particularly suitable for the growth of many other microorganisms. Thus, during commercial yeast manufacture stringent precautions must be taken to avoid this type of contamination by maintaining very high standards of hygiene.

Yeast is easily susceptible and easily suppressed by wild yeasts, moulds and bacteria; its cultivation demands the highest degree of bacteriological cleanliness and sterility in everything it is brought in contact (Walter, 1953). It is well documented that unclean equipment surfaces can harbor various microorganisms (Hobbs, 1974; Hood and Zottola, 1997; Kumar and Anand, 1998). Microorganisms are capable of attaching to processing equipment surfaces used in the food industry forming biofilms which could lead to product contamination with undesirable microorganisms, resulting in spoilage or transmission of disease (Andrade *et al*., 1998; Hassan *et al*., 2004). The processing equipment used in the commercial manufacturing of yeast is complex and often has inaccessible parts which are hard to clean and sanitize effectively. Microorganisms may grow in various niches in fermenters, pipelines, valves, heat exchangers or on equipment surfaces. Preventative measures include effective cleaning and sanitation of equipment.

The application of suitable cleaning agents is therefore essential in order to inactivate any contaminants present in the vessels, pipelines and fittings used in the commercial manufacture of yeast (Walter, 1953). Traditional methods of cleaning yeast processing equipment include steam sterilization and surface sanitation. However, the processing of a highly perishable product, such

as commercial yeast, may require that equipment and product surface areas be cleaned before and after every batch, in which case clean-in-place (CIP) systems may be economically feasible (Troller, 1983), whilst reducing the chances of contamination. CIP systems have become increasingly popular with the food industry because they are efficient and less costly than the more labour-intensive manual cleaning procedures (Troller, 1983). In commercial yeast manufacturing, all fermentation tanks are cleaned with hot alkaline solutions or detergents with CIP equipment (Reed and Nagodawithana, 1991). CIP equipment utilizes a central metering pump, detergent or sanitizer source, and a piping system, which distributes the cleaning solutions to remote locations within the processing plant (Troller, 1983). In the commercial manufacture of yeast, all processing pipes, pumps and valves are cleaned with CIP equipment before the yeast is passed through the process.

MICROBIOLOGICAL CONTAMINANTS OF COMMERCIAL YEAST

The quality of commercial yeast depends not only on viability and fermentation activity, but also on the absence of gross contamination with other microorganisms (Reed and Nagodawithana, 1991). Commercial yeast production is not a completely aseptic process, often resulting in yeast product containing lactic acid bacteria (LAB), wild yeasts and moulds (Reed and Nagodawithana; 1991, Viljoen and Lues, 1993).

Wild yeasts and moulds as contaminants of commercial yeast

Yeasts and molds can be found in a wide variety of environments due to their capacity to utilize a variety of substrates and their tolerance of low pH values, low a_w values, and low temperatures (Membré *et al*., 1999). The term “wild yeast” refers to any other yeast that occurs in fermentation and processing of yeast product other than the culture strain used at the start of fermentation (Tracey *et al*., 1984; Reed and Nagodawithana, 1991). Wild yeasts may enter the commercial yeast factory with the air, the water, and other raw materials, or they may be carried in by insects (Reed and Nagodawithana, 1991). The conditions under which *S. cerevisiae* is propagated are also favourable for the growth of wild yeasts, which even at low levels can adversely affect the gassing power, stability and shelf-life of commercially manufactured yeast (Reed and Nagodawithana, 1991). Baker's yeast production is concerned with spoilage-causing wild yeast

contamination, particularly with species such as *Candida*, *Torulopsis* (Reed and Nagodawithana, 1991), *Geotrichum* and *Hansenula* (Reed and Nagodawithana, 1991).

Air-borne contamination, especially with mould spores, becomes an increasing problem after the liquid yeast is passed through the separators (Irvin, 1954). Many moulds (e.g. *Penicillium* and *Fusarium*) will grow readily on the surface of the yeast cake if not stored at low temperatures (Irvin, 1954; van Dam, 1986). However, moulds grow slowly in comparison to wild yeasts and bacteria, and therefore usually do not present problems in commercial yeast production, unless the product is stored for excessively long periods of time or at elevated temperatures.

Bacteria as contaminants of commercial yeast

Bacterial counts obtained from finished compressed yeast product generally fall between 10^4 and 10^8 bacteria per gram (Reed and Nagodawithana, 1991; Viljoen and Lues, 1993), which predominantly belong to the heterofermentative lactic-acid producing genus *Leuconostoc* or to the homofermentative bacteria of the genus *Lactobacillus* (Reed and Nagodawithana, 1991). Other bacterial contaminants isolated from compressed yeast that belong to the LAB group include *Pediococcus*, *Lactococcus* (Lues, 1992) and *Enterococcus* (*Ent.*) (O'Brien *et al*., 2004a). In general, LAB can grow over a wide temperature and pH range (Franz *et al*., 1991), surviving both the processing and storage of commercial yeast (Viljoen and Lues, 1993). The types of LAB found as contaminants in the compressed yeast industry are most active up to *ca*. 50°C (Walter, 1953).

LAB may have negative effects on both the production and quality of commercial yeast by producing slime or gum which adversely affects the gassing power of the yeast (Tessendorf, 1991). However, contamination of yeast with LAB may reduce the pH of the yeast product, making conditions less favorable for the growth of Enterobacteriaceae and potential foodborne pathogens. The metabolic activity of LAB during bread making has also contributes positively to leavening and flavour of bread (Reed and Nagodawithana, 1991; Enfors, 2001).

Enterococci also belong to the group of LAB and are facultatively anaerobic, catalase negative, Gram-positive cocci arranged in pairs or short chains (Manero and Blanch, 1999). *Enterococcus*

spp. are resilient organisms and are characterized by their ability to survive and multiply in the presence of 6.5% NaCl, 40% bile salt, at 10°C or 45°C, and at pH 9.6 (Morrison *et al*., 1997; Bascomb and Manafi, 1998). Although the majority of *Enterococcus* spp. inhabit the intestines of humans and other animals, some have been found free-living in soil, water, on plants or in dairy products (Manero and Blanch, 1999; Gelsomino *et al*., 2002). In food microbiology *Ent. faecalis* and *Ent*. *faecium* are the most commonly encountered (Hartman *et al*., 1992). In general, *Ent*. *faecalis* is regarded as indicator of contamination from human sources, whereas *Ent*. *faecium* and other species indicate contamination from non-human sources (Reuter, 1992). *Enterococcus* spp. are among the most thermotolerant of non-sporulating bacteria (Franz *et al*., 1999), which results in their contamination of many fermented food products (Devriese *et al*., 1991; Hartman *et al*., 1992; Birollo *et al*., 2001). Due to their ability to survive under adverse environmental conditions, they serve as a good index of hygiene and processing conditions (Hartman *et al*., 1992; Devriese *et al*., 1995; Gelsomino *et al*., 2002). There is a need to determine the source of enterococci in commercially manufactured yeast as they may indicate the presence of other spoilage or pathogenic contaminants (Birollo *et al*., 2001).

Enterococcus spp. are also relatively resistant to chilling (Hartman *et al*., 1992), and can be expected to survive the storage of commercial yeast products at low temperatures. In addition to their spoilage potential of food and beverage products, *Ent*. *faecalis* and *Ent*. *faecium* have been suspected as the causative agents of food borne illnesses, and have been classified as potential emerging food pathogens (Morrison *et al*., 1997; Franz *et al*., 1999), receiving increased attention due to their potential role in serious human infections, such as endocarditis and bacteremia (Knudtson and Hartman, 1992). Another concern and a contributing factor to the pathogenesis of enterococci is their emerging intrinsic resistance to a wide variety of antibiotics including penicillins, sulphonamides, carbapenems and other β-lactams; resulting in community-acquired and in hospital-acquired (nosocomial) infections (Morrison *et al*., 1997; Franz *et al*., 1999).

Indicator microorganisms, such as coliforms, have been found to be suitable indicators of hygiene in fermented products such as sweetened yoghurt (pH 4 – 5), as long as the counts are determined in the first days after production (Birollo *et al*., 2001). Similar to yoghurt, commercial yeast has a pH of 4 – 5 and contains high numbers of LAB, thus coliform organisms and

Escherichia (*E.*) *coli* which have occasionally been found in commercial yeast (Reed and Nagodawithana, 1991; O'Brien *et al*., 2004a) can also be used as indicators of hygiene.

Coliforms are defined as facultatively anaerobic, Gram-negative, rod-shaped bacteria (Prescott *et al*., 1996, Birollo *et al*., 2001). Some coliforms are found in the intestinal tract of man and are indicators of fecal contamination, but most are found throughout the environment and have little sanitary significance (Greenberg and Hunt, 1985). The coliform group includes species of the genera *Escherichia, Klebsiella, Enterobacter* and *Citrobacter* (Prescott *et al*., 1996). Their presence in ready to eat foods is highly undesirable as they may indicate poor manufacturing practices and low hygiene standards (Jay, 1998). Screening food products for coliforms is not intended to detect fecal pollution but rather to measure the quality of the practices used to ensure proper processing and to minimize bacterial contamination on processed products (Birollo *et al*., 2001).

E. coli is an oxidase negative, glucose fermenting bacterium belonging to the coliform group (Prescott *et al*., 1996). *E. coli* is commonly associated with soil, water, plants and the gastrointestinal tracts of humans and animals, and is used as an indicator of food safety (Jay, 1998). The presence of *E. coli* in processed products may indicate the presence of enteric pathogens such as *Salmonella* and *Shigella* (Troller, 1983). However, some have argued that the role of *E. coli* as an indicator organism should be subjugated to its role as a pathogen (Troller, 1983). *E. coli* is easily destroyed by heat (greater than 45°C) and is usually inactivated during freezing and cold-storage (Shapton and Shapton, 1991; Prescott *et al*., 1996). Temperature is the main measure used to retard the growth of *E. coli* in Baker's yeast products by cold storage inactivation at 1 – 5°C.

Several studies (Collins *et al*., 1991; Bailey and von Holy, 1993; Viljoen and von Holy, 1997) have provided evidence that Baker's yeast can contain *Bacillus* spores and thus could serve as a vehicle for entry of *Bacillus* spores into baking environments. *Bacillus* strains are the causative agents of rope-spoilage of bread which is characterized by a sticky crumb and an odour like rotting pineapples (Kirschner and von Holy, 1989; Doyle *et al*., 1997, Viljoen and von Holy, 1997). Spores of *Bacillus* spp. are heat resistant and survive the baking process 200 - 300°C (Pattison, 2000), and after cooling of the bread, the spores germinate and grow as vegetative cells

producing enzymes, which destroy the structure of bread (von Holy and Kirschner, 1991; Bailey and von Holy, 1993). Rope-spoilage caused by *Bacillus* species, occurs mostly in wheat breads containing high concentrations of sugar, fat and/ or fruits (Doyle *et al*., 1997). Although the addition of preservatives, such as calcium propionate in bread can be effective against mould as well as *Bacillus* species that cause rope (Doyle *et al*., 1997; Pattison, 2000), the presence of rope-causing spores in Baker's yeast as an ingredient in commercial bread production is undesirable and should be minimized.

RESEARCH MOTIVATION

The commercial production of yeast for baking and brewing has become a science, consistently searching for ways of improving the efficiency of the process as well as producing yeast of a consistent quality. In the past 20 years, there has been a strong trend towards automation of the commercial yeast industry, which has improved product consistency and quality. However, due to strongly increasing demands with regard to the microbiological quality of commercially manufactured yeast, the prevention of microbial contamination has become of utmost importance. To date, there are few reports concerning the bacterial contamination of commercially manufactured yeast and the effect of these contaminants on product quality and safety for baking and brewing purposes. Since the commercial yeast manufacturing process consists of a number of fermentations, any bacterial contamination in the early stages of manufacture could infiltrate the next phase.

The contamination and spoilage of commercially manufactured yeast by bacteria has not been quantified by the South African yeast manufacturing industries. The first step in controlling or minimizing contamination is to identify the possible source(s) or sites of contamination. Contamination studies can be performed to identify contamination routes and sites in food processing plants to gain information on how to prevent future product contamination. In order to pinpoint initial processing sites as sources of contamination, contaminants should be detected as early as possible in processing. Our study made use of a novel technique, preliminary incubation, which aimed to boost counts for the early detection of bacterial contaminants. This work evaluated a typical commercial yeast factory for sources of bacterial contamination and quantified the bacterial populations associated with the manufacture of cream, compressed and dry yeast products. In addition, this work assessed finished cream, compressed and dry yeast

products for bacterial pathogens: *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and bacterial spores, whose presence could compromise the safety of yeast as a raw material in baking and brewing industries.

The contamination of commercial yeast products with spoilage bacteria during processing could also lead to a shorter shelf-life which could result in economic losses to manufacturers and consumers. The effect of storage temperature and time on the bacterial ecology and shelf-life of commercially manufactured cream, compressed and dry yeast products was also investigated.

Reportedly, unclean food contact surfaces in the food processing environment may act as sources of contamination of passing product (Gibson *et al*., 1999). In the commercial manufacturing of yeast, processing equipment may facilitate contamination of yeast product by microorganisms and even favour their growth. An *in vitro* study to assess the influence of yeast product build-up on processing equipment surfaces on the attachment and possible biofilm formation by bacterial contaminants was also investigated.

The increasing importance of bacteriological standards of commercially manufactured yeast for bread making and brewing emphasizes the need for a full review of existing commercial yeast manufacturing plants from a hygiene point of view. This shall be the focus of this study. This study aimed to determine the factors that influence the quality, safety and shelf-life of commercially manufactured yeast by investigating the product and processing plant for the presence of indicator, pathogenic and spoilage bacteria, respectively.

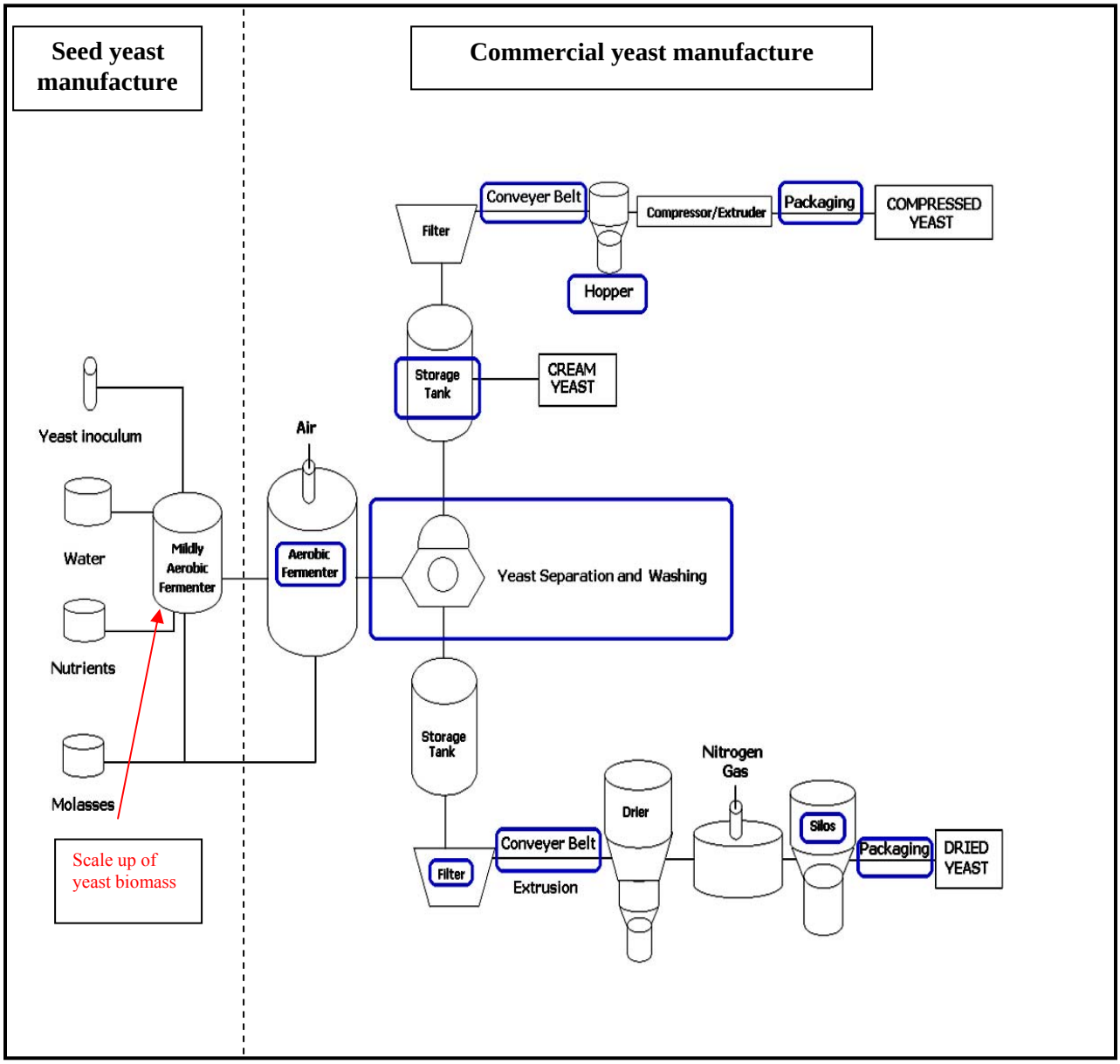


Figure 1.1 Schematic diagram of the commercial yeast manufacturing process

Table 1.1 Typical stages in the propagation commercial yeast (after van Dam, 1986; Reed and Nagodawithana, 1991; Tessendorf, 1991)

Fermentation Stage	**PC 1**	**PC 2**	**PC 3**	**F1**	**F2**	**F3**	**F4**	**F5**
Yeast inoculum	0.2g	0.8g	3.5kg	25kg	120kg	420kg	833kg	1666kg
Total yeast yield	0.8g	3.5kg	25kg	120kg	420kg	2500kg	5000kg (3x)	1 1000kg (3x)
Length of fermentation (h)	24 – 36	24	19	12 – 14	8 – 10	14	14	14 – 15
Temperature	25 – 35	→						
pH	4	→						6

PC: Pure culture, batch fermentation
F1, F2: Batch fermentation
F3, F4, F5: Fed-batch fermentation

CHAPTER TWO

ANALYSIS OF A COMMERCIAL YEAST PRODUCTION PROCESS

CHAPTER 2.1

PROCESSING SOURCES OF BACTERIAL CONTAMINANTION IN CREAM, COMPRESSED AND DRY YEAST PRODUCTION

ABSTRACT

This study evaluated a local commercial yeast factory for sources of bacterial contaminants and further investigated the effect of various processing stages on bacterial numbers. During three replicate surveys, seed, dry, cream and compressed yeast samples emanating from the same batch, as well as corresponding raw materials, were collected from various processing sites. Samples were analysed on the day they were collected (non-PI) and on the day following a preliminary incubation (PI) of 24h at 37°C. The PI procedure was incorporated for amplification of bacterial counts below the lower detection limit (< 0.70 log CFU/ ml). For all samples, the numbers of aerobic bacteria, *Enterococcus*, coliforms and *E. coli* were quantified by standard plate counting procedures on various selective media. In general, raw materials were negligible in contributing to the bacterial contamination of commercial yeast, with the exception of the soda ash used to control the fermentation pH, which contained *ca*. 2 log CFU/ ml *Enterococcus* and aerobic bacteria. The PI technique helped to identify sources of bacterial contaminants, finding the scale up of yeast biomass during seed yeast production to be the initial site of product contamination with *Enterococcus* and aerobic bacteria. In the production of cream and compressed yeast, storage tanks were found to be the initial processing site of contamination with *E. coli* and coliforms, as well as a site for the proliferation of *Enterococcus* and aerobic bacteria. In addition, the hopper used in the mixing and extrusion of yeast cake into 5 kg compressed yeast blocks significantly increased coliform counts. In the production of dry yeast, coliforms and *E. coli* were found in the early stages of commercial fermentation and increased upon the subsequent storage and packaging of dry yeast. In general, coliform and APC for finished cream, compressed and dry yeast product were within the recommended guidelines for use in baking and brewing, however, *E. coli* and *Enterococcus* counts were not. The presence of indicator organisms such as coliforms, *E. coli* and *Enterococcus* highlighted the need for stringent cleaning and sanitation regimes for large-scale fermentative processes such as those used in the commercial yeast factory investigated in this study.

INTRODUCTION

The technology of commercial yeast production is well established and there is a vast body of literature covering production and use thereof (Legan and Voysey, 1991). In brief, commercial yeast is propagated under precisely defined and controlled conditions having four stages: (1) fermentation (growth of yeast), (2) separation (concentration of yeast), (3) dehydration (by filtration and/ or drying) and (4) packaging (Pattison and von Holy, 2001). The most widely recognized role of yeast is as a leavening agent in bread and other fermented products (Legan and Voysey, 1991).

Yeast intended for commercial use is a product featuring many quality parameters from baking or brewing performance to microbiological standards. Commercial yeast is not propagated under pure culture conditions in the commercial fermentation stages (Tessendorf, 1991). In addition, the commercial fermentations are carried out in large fermentation vessels that logistically cannot be kept completely sterile, making it almost impossible to avoid some form of microbial contamination (Reed and Nagodawithana, 1991; Barrette *et al*., 1999). Although great efforts are made to keep microbial contamination to a minimum, various microbial contaminants are contained within finished commercial yeast products (Reed and Nagodawithana, 1991). Post-fermentation processing consists of a number of stages, which can also lead to increases in the microbial contamination or proliferation of contaminants within the product e.g. washing and separation, dehydration, packaging, storage and transportation (Lues, 1992).

Not all microorganisms flourish under the same environmental conditions, thus only certain groups of microorganisms are capable of contaminating yeast in its different stages of production. Numerous studies have reported bacterial contaminants of commercial yeast products include predominantly lactic acid bacteria (LAB) (Reed and Nagodawithana 1991; Lues, 1992) and occasionally *Enterococcus* spp. (O'Brien *et al*., 2004a), *Bacillus* spores (Bailey and von Holy, 1993; Viljoen and von Holy, 1997) as well as coliforms and *E. coli* (Reed and Nagodawithana, 1991; O'Brien *et al*., 2004a). Possible sources of contamination previously identified include raw materials; processing water; chemicals (defoamer); insufficient cleaning of fermentation tanks,

storage tanks, dryers, filter cloths and product contact surfaces; airborne contamination or unhygienic handling of yeast products (Tessendorf, 1991; Lues, 1992).

The raw materials used in the manufacture of commercial yeast are rich in microbial nutrients and are thus excellent substrates for microbial growth. Before the molasses can be used for the cultivation of yeast it must be treated (Walter, 1953), in order to eliminate any contaminating microorganisms or substances that may inhibit the growth of the yeast or impair its' function and/ or quality. The molasses, which may contain high numbers of microorganisms, is sterilized at high temperature (± 107°C) after dilution and standardization of the sugar concentration before entering the process as sterile molasses, thus minimizing the introduction of microbial contaminants (Reed and Nagodawithana, 1991; Enfors, 2001).

Aerobic industrial fermentation processes require a system for aeration and mixing of the culture (Ward, 1992). In the commercial yeast manufacturing process, an adequate supply of air is essential for maximum yeast cell growth (Walter, 1953). Typical air-borne microorganisms are bacterial and mold spores, wild yeasts and LAB, which are undesirable contaminants in the commercial manufacturing of yeast. Thus, the air for the commercial yeast process is filter sterilized (Reed and Nagodawithana, 1991; Enfors, 2001). The sterile air is introduced at the base of the tank and effectively dispersed throughout the medium by an agitation system (Ward, 1992). The large volume of air used in aerobic fermentation requires the use of defoaming agents (Reed and Nagodawithana, 1991), which could subsequently also be a source of contamination in the commercial manufacture of yeast.

Since the conditions under which the yeast are grown are also particularly suitable for the growth of many other microorganisms, the process is run at pH 4 - 5 with a large inoculum and short fermentation time, which helps to prevent contaminating microorganisms from proliferating or out-competing the yeast (Enfors, 2001). The pH is an important parameter for controlling the growth of contaminants in yeast fermentations, since yeast are capable of growth at much lower pH values than most bacteria (Reed and Nagodawithana, 1991). The pH values in the acidic range reduce bacterial contamination but tend to increase adsorption of molasses colouring by the yeast cells. Thus, most commercial fermentation stages start at lower pH values of 4.0 – 4.4 and

end at a higher pH range of 4.8 – 5.0 (Ward, 1992). The pH is constantly monitored and controlled by the addition of soda ash or sulphuric acid (H_2SO_4).

In the manufacturing of commercial yeast, process water is required in the dilution of molasses, in fermentations, during separation and washing, and for cleaning purposes. Thus, the efficient chlorination of water is an important step in reducing the contamination of commercially manufactured yeast. Chlorine is widely used for the disinfection of water (Troller, 1983), which may contain a variety of waterborne bacteria such as coliforms (Prescott *et al*., 1996).

The movement of product from one area of operation to another is a highly critical portion of any food manufacturer's process line (Troller, 1983). If perishable, product conveying must not be delayed or it could spoil or become a potential health risk (Troller, 1983). Since yeast is an excellent nutrient source for bacteria, any deposits of wet or dry yeast on processing equipment surfaces become potential sites for contamination (Reed and Nagodawithana, 1991). Thus, effective cleaning and sanitation of all process equipment (e.g. fermentation vessels, separators, filter presses, dryers and packaging equipment) is required in order to help reduce the levels of microbial contaminants.

With an increase in the export of locally manufactured commercial yeast, the increasing focus is on contamination with bacteria such as *E. coli*, coliforms and *Enterococcus* spp. As a raw material in the baking and brewing industry, Baker's and Brewer's yeast must comply with desired guidelines (SABS 1642, 1995; SABS 1643, 1995). Standards for edible compressed yeast and dried yeast that is intended for use in baking and in the brewing of beer, respectively, states that yeast shall be a substantially pure culture of an acceptable strain of *Saccharomyces* (*S*.) *cerevisiae*, with a characteristic yeasty odour and taste, with no signs of deterioration or decomposition. Guidelines for yeast used in baking restrict the number of viable bacteria to less than 9 log CFU/ g, coliform organisms to less than 2 log CFU/ g, and *E. coli* and *Salmonella* absent in 1 g and 25 g, respectively (SABS 1642, 1995). Guidelines for yeast used in brewing restrict the number of viable bacteria to less than 8.5 log CFU/ g, coliform organisms to less than 2 log CFU/ g, and *E. coli* and *Salmonella* absent in 1 g and 25 g, respectively (SABS 1643, 1995). Limits for *Enterococcus* spp. in Baker's yeast have not yet been established due to the

largely unknown implications of its use as a raw material if contaminated; however local and international guidelines for Brewer's yeast restrict the number to less than 1.7 log CFU/ g.

Due to increasing demands with regard to the microbiological quality of commercially manufactured Baker's and Brewer's yeast, this study investigated a typical commercial yeast factory for potential sources of *E. coli*, coliforms, *Enterococcus* and aerobic bacteria, and examined the influence of various stages of processing on the bacterial contamination and subsequent numbers in the finished cream, compressed and dry yeast products.

MATERIALS AND METHODS

(a) Raw material and commercial yeast process analysis

Three independent replicate surveys of a commercial yeast production plant were conducted over 6 months, from July to December 2003. During surveys, 2 pre-treated raw materials (molasses and synthetic sugar), 9 raw materials and 1 process air, 7 seed yeast, 1 cream yeast, 8 compressed yeast, and 8 dry yeast production line samples were aseptically collected (Figure 2.1.1 and Table 2.1.1).

(b) Sample collection

For each survey, raw material (Figure 2.1.2) and seed, cream and compressed and dry yeast production line samples corresponding with the same fermentation and/ or batch were aseptically collected. For process air analysis, incoming air was sampled after absolute filtering and upon entry into commercial fermentations by means of a sterile air sampler (Figure 2.1.2, sample R 12), aseptically connected to the fermentation tank. The air sampler holds six plates (standard 90 mm Petri dishes) where duplicate plates of three selective media types (Figure 2.1.3 and Table 2.1.2) were placed inside the air sampler and exposed to the incoming air for 20 min. Liquid samples were aseptically sampled directly into sterile Whirl Pak bags (Nasco, U. S. A.) by means of tank and/ or pipeline sampling valves, which were steam sterilized and/ or sprayed with 70% alcohol before sampling. For solid samples, aliquots were aseptically collected using sterile

disposable gloves (Union Drug, Canelands, South Africa) and placed into sterile Whirl Pak bags (Nasco). All samples were kept on ice and analysed within 24 h. Duplicate plates used for process air analysis were incubated immediately after transport.

(c) Sample processing and analysis

All samples were analysed on the day of sampling and again after preliminary incubation (PI) at 37°C for 24h, which aimed to boost bacterial counts before analysis (O'Brien *et al*., 2004a). PI samples were analysed in the same manner as the corresponding non-preliminary incubated (non-PI) samples.

Samples were prepared for analysis according to the sample consistency (liquid, solid, dry) (Table 2.1.1). For liquid samples, 10 ml aliquots were aseptically removed from the original sample and homogenized for 2 minutes using a Colworth 400 Stomacher (Seward Medical, London, UK) before analysis. For solid samples, 10 g aliquots were aseptically removed from the original sample and homogenized in 90 ml Tryptone Soya Broth (TSB) (Oxoid, Basingstoke, UK) and homogenized as above. For dry yeast samples, 10 g aliquots were aseptically removed from the original sample and incubated in 90 ml TSB (Oxoid) at 37^{o}C for 30 min, and homogenized as described above. The 30 minute incubation step facilitated the rehydration of yeast cells as well as the recovery of any contaminants present before analysis (O'Brien *et al.*, 2004a).

Following homogenization, samples were analysed for *E. coli*, coliform, *Enterococcus* and aerobic plate counts (APC), by preparing single ten-fold dilutions in sterile buffered peptone water (BPW) (Oxoid) which were plated in duplicate using standard pour- and spread-plate techniques on various selective media (Figure 2.1.3 and Table 2.1.2). For raw material samples, 1 ml spread plates of the first dilution (neat or 10^{-1}) were also prepared in duplicate for the detection of low numbers of bacteria in APC. After incubation (Figure 2.1.3 and Table 2.1.2), plates showing between 30 and 300 colonies (or the highest number if below 30) were counted and the log colony forming units (CFU) per ml or per g determined. In addition, the pH for both

non-PI and PI samples was measured at 25°C with a Metrohm 744 pH meter (Metrohm Ltd., Herisau, Switzerland).

(d) Statistical analysis

Counts and pH measurements obtained for each sample for the three replicate surveys were meaned and standard deviations between samples calculated. Standard deviations between the same sample types were calculated in order to assess the variability between the three surveys. In addition, colony count data and the pH of samples were statistically analysed using the analysis of variance (ANOVA) test at the 95% confidence level using the STATGRAPHICS (STSC Inc. and Statistical Graphics Corporation) programme.

RESULTS

Results obtained during the 3 replicate surveys of a commercial yeast process are shown in Figure 2.1.4 to Figure 2.1.6. Means, standard deviations and significant differences ($P < 0.05$) of pH, aerobic, *Enterococcus*, coliform and *E. coli* counts for seed yeast production (Figure 2.1.4), cream and compressed yeast production (Figure 2.1.5) and dry yeast production (Figure 2.1.6) are shown before and after preliminary incubation of samples.

In general, the standard deviations obtained, suggested the bacterial counts were not significantly different ($P < 0.05$) between the 3 replicate surveys conducted over 6 months. In addition, for most samples, where present, aerobic plate counts were consistently higher than *Enterococcus*, coliform and *E. coli* counts, respectively. Similarly for most samples, bacterial counts for PI samples were significantly higher ($P < 0.05$) than non-PI samples. Particularly, aerobic plate and *Enterococcus* counts, which in most samples increased by 2 – 4 log CFU/ ml or g after preliminary incubation at 37°C for 24h. Mean finished product counts and standard deviations for cream, compressed and dry yeast samples before and after preliminary incubation at 37°C are shown in Table 2.1.3.

(a) Bacterial counts associated with raw materials

In general, aerobic plate, *Enterococcus*, coliform and *E. coli* counts for raw materials were below the lower detection limit (< 0.7 log CFU/ g or ml) even after preliminary incubation, except those of soda ash which contained *ca.* 2 log CFU/ ml *Enterococcus* and corresponding aerobic bacteria. Results showed the sterilization of raw molasses before entry into yeast fermentations as sterile molasses, decreased aerobic and *Enterococcus* counts (*ca.* 2 log CFU/ ml) to below the detection level of 0.7 log CFU/ ml. Average pH measurements for raw materials were synthetic sugar (pH 4); mineral mix, raw molasses, vitamin mix and yeast inoculum (pH 5); compressed and dry yeast emulsifier (pH 6.5); defoamer and process water (pH 8); soda ash (pH 11.5). Measurements of pH before and after preliminary incubation of raw material samples were not significantly different ($P > 0.05$).

(b) Bacterial counts associated with seed yeast production

Even after preliminary incubation at 37°C for 24 h, aerobic bacteria, *Enterococcus*, coliforms and *E. coli* were non-detectable (< 0.70 log CFU/ ml) during the early stages of seed yeast production (Figure 2.1.4, sample S1, S2 and S3). Both non-PI and PI counts found the Mother fermentation (MF), the final fermentation during seed yeast manufacturing, to be the initial site of contamination of the seed yeast with *Enterococcus* and corresponding aerobic bacteria (*ca.* 1 log CFU/ ml) (Figure 2.1.4, sample S4). Following separation and acid treatment the resultant seed used to start commercial fermentations in the production of cream, compressed and dry yeast contained *ca.* 1.5 log CFU/ ml *Enterococcus* and aerobic plate counts (Figure 2.1.4, sample S7). *E. coli* and coliforms were not detected throughout the production of seed yeast. This result was confirmed by the use of the preliminary incubation technique, which found coliforms and *E. coli* to be absent in all seed yeast samples. The mean pH of seed yeast samples was *ca.* 4.8 (Figure 2.1.4).

(c) Bacterial counts associated with cream and compressed yeast production

Initial counts for *Enterococcus* and aerobic bacteria were significantly lower ($P < 0.05$) in commercial fermentations during the production of cream and compressed yeast compared to dry yeast (Figure 2.1.5, sample C1). Results showed a significant increase ($P < 0.05$) in *Enterococcus* and aerobic counts following the storage of yeast cream and further processing into yeast cake. Both non-PI and PI counts confirmed the storage of yeast cream (Figure 2.1.5, sample C4) as the initial site of contamination with *E. coli* and coliforms (*ca.* 1 and 2 log CFU/ ml and g, respectively). The mixing of yeast cake in the hopper and extrusion into 5 kg compressed yeast blocks significantly increased ($P < 0.05$) coliform counts by 1 log CFU/ g (Figure 2.1.5, sample C7). Following filtering of yeast cake, there appears to be a "step-wise" relation between *E. coli*, coliforms, *Enterococcus* and aerobic bacteria, respectively (Figure 2.1.5, sample C6 to C9). *Enterococcus* and aerobic counts for finished cream and compressed yeast product were *ca.* 4 and 5 log CFU/ ml and g, respectively (Figure 2.1.5, sample C5 and C9, Table 2.1.3). The mean pH of cream and compressed yeast samples was *ca.* 4.9 (Figure 2.1.5).

(d) Bacterial counts associated with dry yeast production

Preliminary incubation established that the start of commercial fermentation in the production of dry yeast was already contaminated with low levels of *E. coli* and coliforms (Figure 2.1.6, sample D1). *E. coli* and coliform counts decreased to below the lower detection limit (< 0.7 log CFU/ g or ml) after separation, cold storage and filtering, however, were re-introduced after extrusion, and during the storage and packaging of dry yeast (*ca*. 2 and 3 log CFU/ g, respectively) (Figure 2.1.6, sample D7 and D8, Table 2.1.3). For contamination with *Enterococcus* and aerobic bacteria, results from this study showed that seeding of the commercial fermentation with already contaminated seed yeast resulted in counts of *ca.* 2 log CFU/ ml. Similar to cream and compressed yeast production, aerobic and *Enterococcus* counts increased significantly to *ca.* 3 log CFU/ ml ($P < 0.05$) after the storage of yeast cream (Figure 2.1.6, sample D4) and to 4 log CFU/ g after the filtering, extrusion and drying of yeast. *Enterococcus* and aerobic plate counts for finished dry yeast product were *ca.* 4 log CFU/ g (Figure 2.1.6, sample D8; Table 2.1.3). The mean pH of dry yeast samples was *ca.* 5.3 (Figure 2.1.6).

DISCUSSION

Previous reports of the presence and possible sources of *Enterococcus*, coliforms and *E. coli* throughout the production of cream, compressed and dry yeast (O'Brien *et al*., 2004a) were confirmed in this study. These bacteria are generally expected as contaminants of fermented products, since the conditions are favorable for the growth of mesophilic, aerobic or facultative anaerobic organisms capable of growing at a pH between 4 and 5 (Lengeler *et al*., 1999), similar to the conditions used in the commercial fermentation of cream, compressed and dry yeast.

In many food and beverage processes, the presence of indicator organisms such as coliforms and *E. coli* is used to assess product quality and processing conditions (Reed and Nagodawithana, 1991; Jay, 1998). Reportedly, coliforms and *E. coli* have been previously used as indicator organisms of hygiene in fermented products, such as yoghurt (Birollo *et al.*, 2001). Given the importance of coliforms and *E. coli* as indicators of failed sanitation (Keller *et al*., 2002), their presence often suggests improved cleaning practices are imperative in reducing contamination with the afore-mentioned indicator organisms as well as possible associated spoilage and/ or pathogenic organisms (Troller, 1983). *Enterococcus* spp. are also a good index of hygiene in food processing due to their ability to survive adverse environmental conditions (Devriese *et al*., 1991; Hartman *et al*., 1992; Giraffa *et al*., 1997), especially in process lines of fermented products (Birollo *et al*., 2001). The presence of enterococci in dairy products, for example milk, has long been considered an indication of insufficient sanitary conditions (Giraffa, 2002). In commercial yeast production, the presence of *Enterococcus* is also undesirable because of its potential role in spoilage and quality defects (Franz *et al*., 1999), either of the yeast itself or of its associated products for which it may be used as a raw material, such as in the baking of bread and fermentation of alcoholic beverages.

In general, this study found raw materials i.e. yeast inoculum, sterile molasses and synthetic sugar mix, defoamer, vitamin mix, mineral mix, process water, emulsifiers and process air to be negligible in contributing to the contamination of commercially manufactured yeast. Molasses, the main raw material used in the commercial propagation of yeast, is a by-product of the sugar industry, which not only supplies the yeast with fermentable sugars as a source of carbon and

energy but also essential growth factors (van Dam, 1986; Reed and Nagodawithana, 1991). The analysis of raw molasses and the synthetic sugar before and after sterilization at ± 107°C for 30 minutes, found the sterilization process reduced *Enterococcus* and corresponding aerobic plate counts from *ca*.1 and 2 log CFU/ ml respectively, to below the lower detection limit (0.7 log CFU/ ml). Thus reconfirming that the sterilization of molasses, a raw material capable of harboring and introducing a variety of spoilage and potentially pathogenic organisms, is essential before entering into fermentation processes (Reed and Nagodawithana, 1991; Enfors, 2001).

The raw material, soda ash, used to control the pH of larger fermentations following and including the Mother fermentation was found to contain *ca*. 2 log CFU/ ml *Enterococcus* and corresponding aerobic bacteria. It has previously been reported that *Enterococcus* spp. are able to grow at high pH up to 9.6 (Devriese *et al*., 1991; Manero and Blanch, 1999). Furthermore, extreme alkaline shock response studies of *E. faecalis* found that cells were tolerant up to pH 11.9 and that 37 stress proteins were amplified (Flahaut *et al*., 1997). Results from our study showed that *Enterococcus* was able to survive in soda ash (*ca.* pH 11.5). We therefore suggest that soda ash may be a possible source of seed yeast contamination with *Enterococcus*. This suggestion is further supported by the observation that PI results in this study showed that the contamination of seed yeast with *Enterococcus* coincided with the use of soda ash during the scale up of yeast biomass to serve as an inoculum for larger commercial fermentations. The high numbers of *Enterococcus* found during the scale up of yeast were expected not only in accordance with previous findings (O'Brien *et al*., 2004a), but also due to the well documented synergistic relationship which exists between yeasts and LAB, a family to which *Enterococcus* spp. belong (Daniel, 1972; Oura *et al*., 1982; Lues, 1992).

In seed yeast production, the addition of phosphoric acid after separation provides the yeast with phosphorus for growth whilst lowering the pH, which aims to reduce the presence and/ or proliferation of any bacterial contaminants. In this study, acid treatment reduced the pH of the seed yeast from *ca*. 5.5 to 3.5, and appeared to significantly ($P < 0.05$) reduce aerobic plate counts by *ca.* 0.5 log CFU/ ml. In general, results from this study found the level of contamination to be relatively small compared to the large number of culture yeasts in seed and commercial fermentations. Reed and Nagodawithana (1991), found during the active stages of

fermentation, the pure culture yeasts that are well adapted to wort as a nutrient generally outgrow any contaminating organisms. Hence, bacterial counts in this study only increased upon subsequent processing of relatively inactive yeast (at 4ºC) into cream, compressed and dry yeast.

The preliminary incubation technique was successful in increasing bacterial counts for the detection of low levels of bacteria and in the identification of sources of contamination during the production of cream and compressed yeast. For example, the PI technique helped to identify the storage of yeast cream in bulk storage tanks as the initial source of coliforms and *E. coli*, and a processing site for the unwanted proliferation of *Enterococcus* and aerobic plate counts in the production of cream and compressed yeast. This result was comparable to previous studies of milk and cheese production processes, in which bulk storage tanks were reportedly a source of bacterial contamination (Yoshida *et al*., 1998; Franz *et al*., 2003). In the commercial production of yeast, all storage tanks were cleaned with hot alkaline detergent solutions with clean-in-place (CIP) equipment every three days. Results from this study therefore suggested that reduced time intervals between cleaning of storage tanks may help prevent the build up of yeast cream debris, which in turn form protected niches which are favorable for the growth of bacterial contaminants.

The hopper, in which filtered yeast cake is mixed with emulsifier and extruded in the form of 5 kg compressed yeast blocks, significantly increased coliform counts by *ca*. 0.5 log CFU/ g. This contamination was not thought to originate from the emulsifier, since the corresponding emulsifiers (R10, R11) used in all three surveys tested negative for the presence of bacterial contaminants. Instead, the increased coliform counts were thought to be either due to contamination from insufficiently cleaned surfaces inside the hopper or due to the proliferation of existing cells in the product during mixing and extrusion which raises the temperature of the yeast cake to *ca.* 7°C making it favourable for the growth and/ or proliferation of contaminants (data not shown).

In dry yeast production, preliminary incubation identified the presence of *E. coli* and coliforms at the start of commercial fermentation, however, became non-detectable after separation of the yeast cream. Similar to cream and compressed yeast production, the storage of yeast cream during dry yeast production significantly increased *Enterococcus* and aerobic plate counts, which

otherwise remained fairly constant throughout the production of dry yeast. The extrusion of filtered cake into 'yeast beard' before drying marked the re-contamination of yeast with coliforms, which subsequently increased upon dry storage and packaging of the dry yeast. Reportedly, coliforms can grow at temperatures as high as 50°C and have been found in a variety of dried food products including dehydrated soup, dried milk and dried egg (Jay, 1998). Coliforms are thus, capable of surviving and proliferating during the drying of yeast at ± 50°C for 30 min.

Previous studies have found packaging can contribute to the bacterial contamination of finished products with Gram-negative bacteria (Dykes *et al*., 1991, Georgnaras *et al*., 1996). In this study, *E. coli* counts increased significantly ($P < 0.05$) from below the lower detection limit (< 0 .70 log CFU/ g) to *ca.* 2 log CFU/ g during the packaging of dry yeast. Possible sources of contamination of the dry yeast product with *E. coli* may be attributed to the packing line equipment (e.g. conveyors and dosing cups), packaging material or the workers.

In addition to the main objective of this study of finding the possible sources of yeast product contamination with *E. coli*, coliforms, *Enterococcus* and aerobic bacteria, this study also evaluated the resulting levels of these contaminants in finished cream, compressed and dry yeast products and their acceptability for use in baking and/ or brewing. For all three product types, total bacterial counts (*ca.* 4.5 – 5 log CFU/ ml or g) were well below the recommended guidelines (*ca.* 7 – 9 log CFU/ ml or g). These counts were comparable to counts obtained by other local yeast manufacturers, since bacterial contamination of Baker's compressed yeast commonly reaches 7 log CFU/ g (Viljoen and Lues, 1993).

However, the presence of *E. coli* in all three yeast products tested in this study exceeded the recommended guidelines for its use for baking and brewing. Coliform counts for cream yeast were within the recommended guidelines for its use in baking, however compressed and dry yeast counts exceeded the recommended guidelines by *ca.* 1 log CFU/ g, for use in baking and brewing, respectively. Similar to previous findings (O'Brien *et al*., 2004a), *Enterococcus* counts did not meet the recommended guidelines (1.7 log CFU/ g) for the use of dry yeast for brewing

and wine production. Specifically, *Enterococcus* counts for dry yeast were *ca*. 1 log CFU/ g higher than corresponding *Enterococcus* counts in a previous yeast study (O'Brien *et al.*, 2004a).

CONCLUSION

This study confirmed existing knowledge, namely that commercial yeast is not a monoculture and harbors bacterial contaminants that could compromise its quality and/ or use as a raw material in the commercial production of bread and/ or fermentation of alcoholic beverages. The preliminary incubation technique used in this study proved to be an innovative and effective method for the detection of low levels of bacteria as well as in its application in finding the possible sources of bacterial contaminants during the manufacturing of cream, compressed and dry yeast. Based on this study, results may provide information to manufacturers on the possible sources and levels of *Enterococcus*, coliforms and *E. coli* throughout the production of Baker's and Brewer's yeast, which could be used to reduce the entry and/ or proliferation of these bacteria in locally manufactured yeast. However, in striving for 'pure' culture yeast, one may increase the numbers of more 'unwanted' Gram-negative flora and potentially pathogenic bacteria, which are largely reduced by competition with contaminants belonging to the LAB family. In this study, the presence of marker organisms, such as *E. coli*, coliforms, and enterococci emphasizes the need for more efficient and frequent cleaning and sanitation regimes used in the commercial production of cream, compressed and dry yeast. Strict control of cleaning at each step of the process is essential with increased attention on the following areas: fermentation tanks, bulk storage tanks, driers and hoppers.

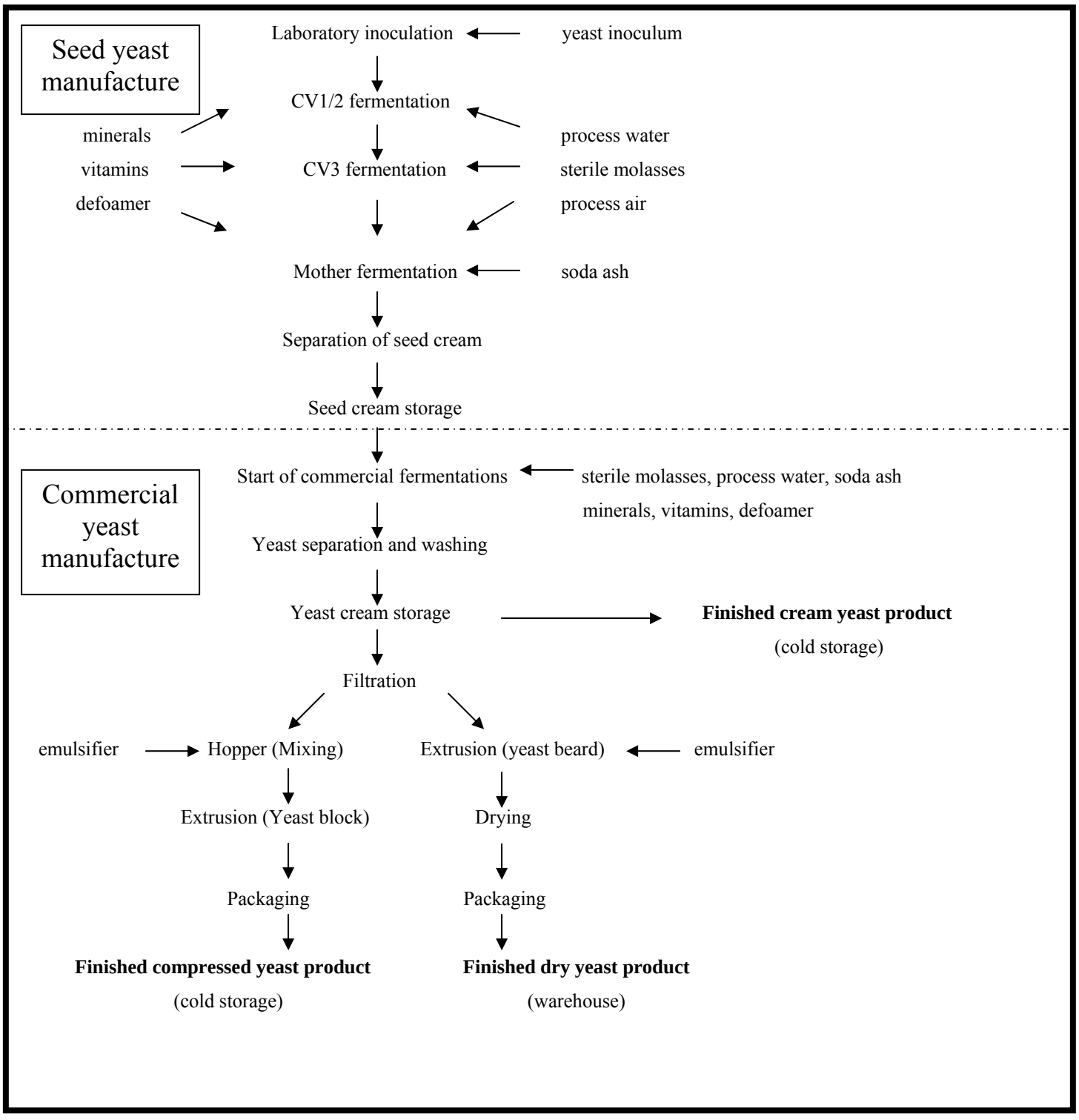


Figure 2.1.1 A commercial yeast manufacturing process with sampling areas for raw material analysis and seed, cream, compressed and dry yeast process analysis

Table 2.1.1 Description of raw material and yeast production line samples

Sample type	Sample Code	Sample description	Sample consistency (liquid/ solid/ dry / air)
Raw Material	R1	Yeast inoculum	Liquid
	R2	Raw molasses	Liquid
	R3	Synthetic sugar[a]	Liquid
	R4	Strongwort[b]	Liquid
	R5	Defoamer	Liquid
	R6	Soda ash	Liquid
	R7	Vitamin mix	Solid
	R8	Mineral mix	Solid
	R9	Process water	Liquid
	R10	Emulsifier – Dry yeast production	Solid
	R11	Emulsifier – Compressed yeast production	Liquid
	R12	Process air[c]	Air
Seed yeast	S1	Start of CV3 after transfer of CV1/ 2	Liquid
	S2	End of CV3 during transfer to Mother Fermentation (MF)	Liquid
	S3	Start of MF after transfer of CV3	Liquid
	S4	End of MF before separation	Liquid
	S5	Seed cream during separation	Liquid
	S6	Seed cream after separation from the liquid yeast vessel (before acid treatment)	Liquid
	S7	Seed cream after separation from the liquid yeast vessel (after acid treatment[d])	Liquid
Cream and compressed yeast	C1	Start of commercial fermentation for cream and compressed yeast production	Liquid
	C2	Weak wort from the fermenter prior to separation	Liquid
	C3	Liquid yeast from the separator	Liquid
	C4	Liquid storage from the storage tank	Liquid
	C5	Finished cream yeast product	Liquid
	C6	Yeast cake from the filter press/ conveyor	Solid
	C7	Yeast cake from hopper prior to forming	Solid
	C8	Block yeast from the packing line prior to wrapping	Solid
	C9	Wrapped compressed yeast product from the product line prior to cold storage	Solid
Dry yeast	D1	Start of commercial fermentation for dry yeast production	Liquid
	D2	Weak wort from the fermenter prior to separation	Liquid
	D3	Liquid yeast from the separator	Liquid
	D4	Liquid storage from the storage tank	Liquid
	D5	Yeast cake from the filter press/ conveyor	Solid
	D6	Extrudate (Yeast beard) from the conveyor line prior to drying	Solid
	D7	Dry yeast storage (silo/ bag) prior to packaging	Dry
	D8	Packed dry yeast product from the packing line prior to warehousing	Dry

[a] Sugar supplement from maize containing 67% sugar, which is used to increase levels of sucrose in larger fermentations
[b] Heat sterilized (107^{o}C for 30 min) mixture of raw molasses and synthetic sugar diluted with process water
[c] Process air was sampled after absolute filtering and upon entry into commercial fermentations (C1 or D1) by means of a sterile air sampler, aseptically connected to the fermentation tank
[d] Seed yeast is treated with phosphoric acid which reduces the pH and thus reduces the growth of any bacterial contaminants present

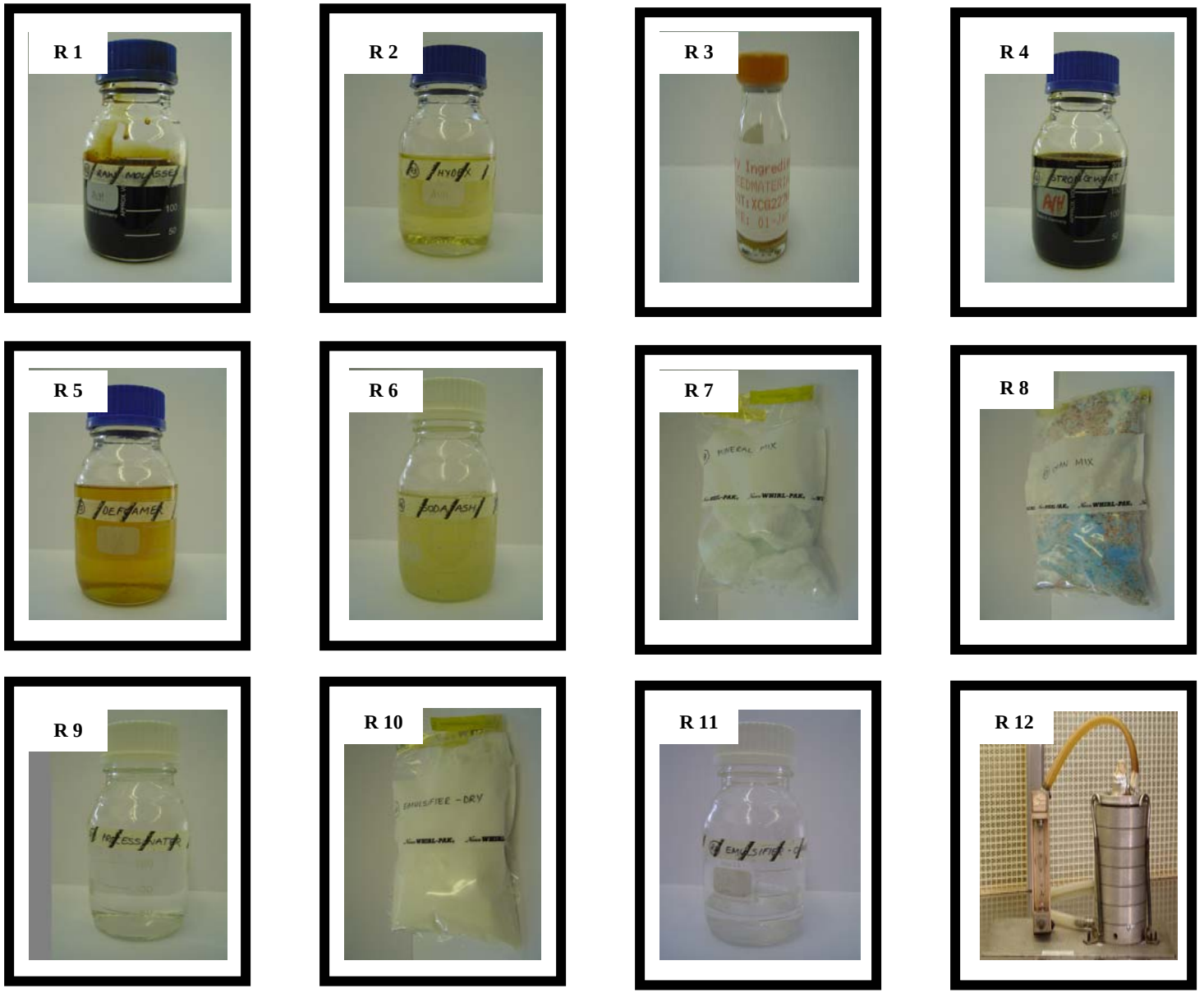


Figure 2.1.2 Pre-treated raw materials [molasses **(R1)** and synthetic sugar **(R2)**]; raw materials [yeast inoculum **(R3)**, sterile molasses **(R4)**, defoamer **(R5)**, soda ash **(R6)**, vitamin mix **(R7)**, mineral mix **(R8)**, process water **(R9)**, emulsifier – dry yeast production **(R10)** and emulsifier – compressed yeast production **(R11)**]; as well as process air **(R12)** were sampled during three replicate surveys of a commercial yeast factory.

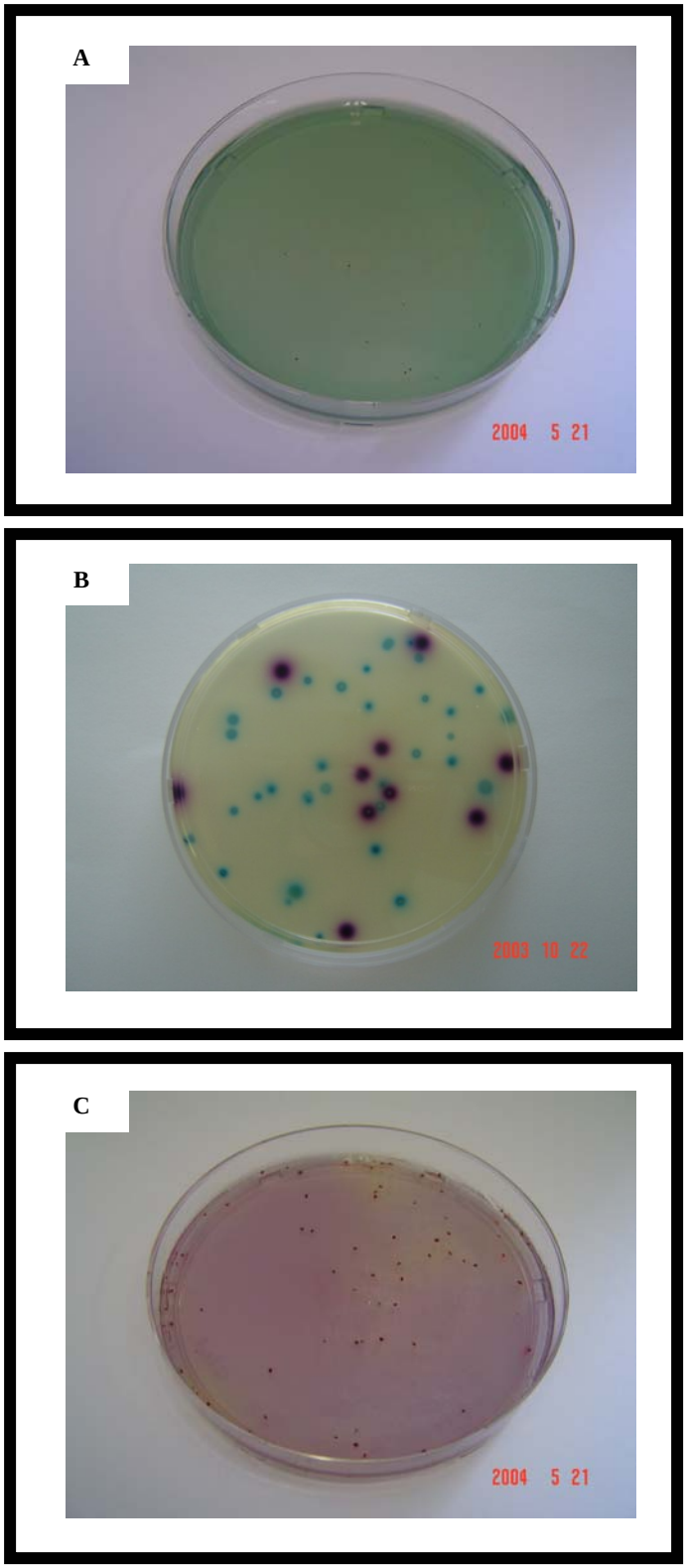


Figure 2.1.3 WL Nutrient agar plus 1% cyclohexamide was used to determine total aerobic plate counts **(A)**, Rapid' *E. coli* 2 agar for coliform (green colonies) and *E. coli* (purple colonies) counts **(B)**, and KF Streptococcus agar reinforced with 1% bacteriological agar plus 1% (v/v) 2-3-5-triphenyl-2H-tetrazolium chloride (TTC) for *Enterococcus* counts **(C)** associated with raw materials and yeast samples.

Table 2.1.2 Media and incubation conditions used for sample analysis

Analysis	Incubation		Growth medium
	Time (h)	Temperature (^{o}C)	
Aerobic plate count	48	30	WL Nutrient Agar (Oxoid) + 1% cyclohexamide (0.004g/ litre) (Scharlau, Barcelona, Spain)
Coliform count and *E. coli* count	24	37	Rapid' *E. coli* 2 Agar (Bio-rad, Marnes-La-Coquette, France)
Enterococcus count	48	37	KF Streptococcus Agar (Oxoid) reinforced with 1% bacteriological agar (Biolab, Midrand, South Africa) + 1% (v/v) 2-3-5-triphenyl-2H-tetrazolium chloride (TTC) (Scharlau)

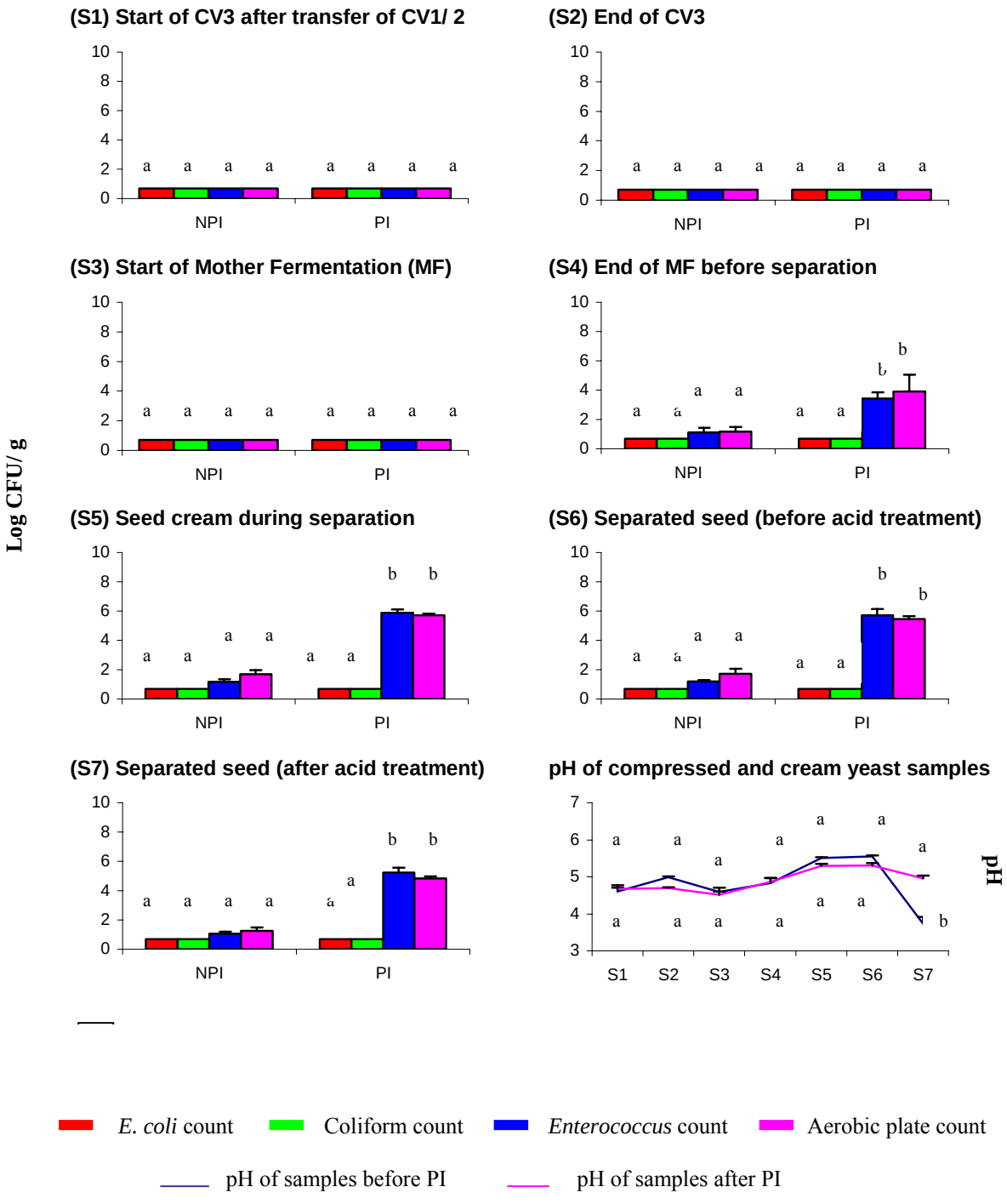


Figure 2.1.4 Mean *E. coli*, coliform, *Enterococcus* and aerobic plate counts (Log CFU/ ml), before and after preliminary incubation at 37°C for 24 h, over three replicate surveys for seed yeast production (Lower detection limit = 0.7 log CFU/ ml). Mean pH over three replicate surveys is shown for seed yeast production. Mean bacterial counts followed by the same superscripts are not significantly different (P > 0.05).

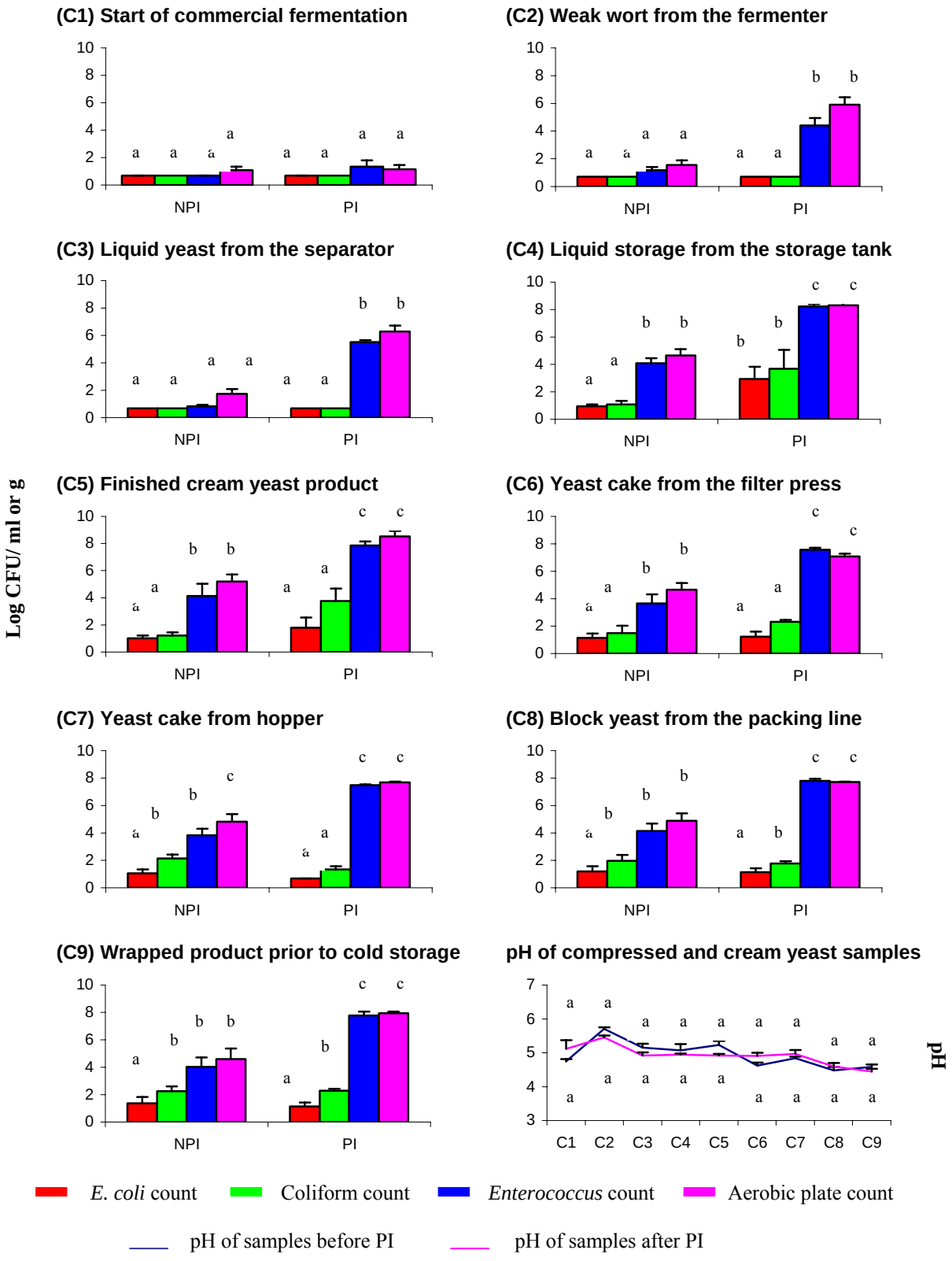


Figure 2.1.5 Mean *E. coli*, coliform, *Enterococcus* and aerobic plate counts (Log CFU/ ml or g), before and after preliminary incubation at 37°C for 24 h, over three replicate surveys for cream and compressed yeast production (Lower detection limit = 0.7 log CFU/ ml or g). Mean pH over three replicate surveys is shown for cream and compressed yeast production. Mean bacterial counts followed by the same superscripts are not significantly different (P > 0.05).

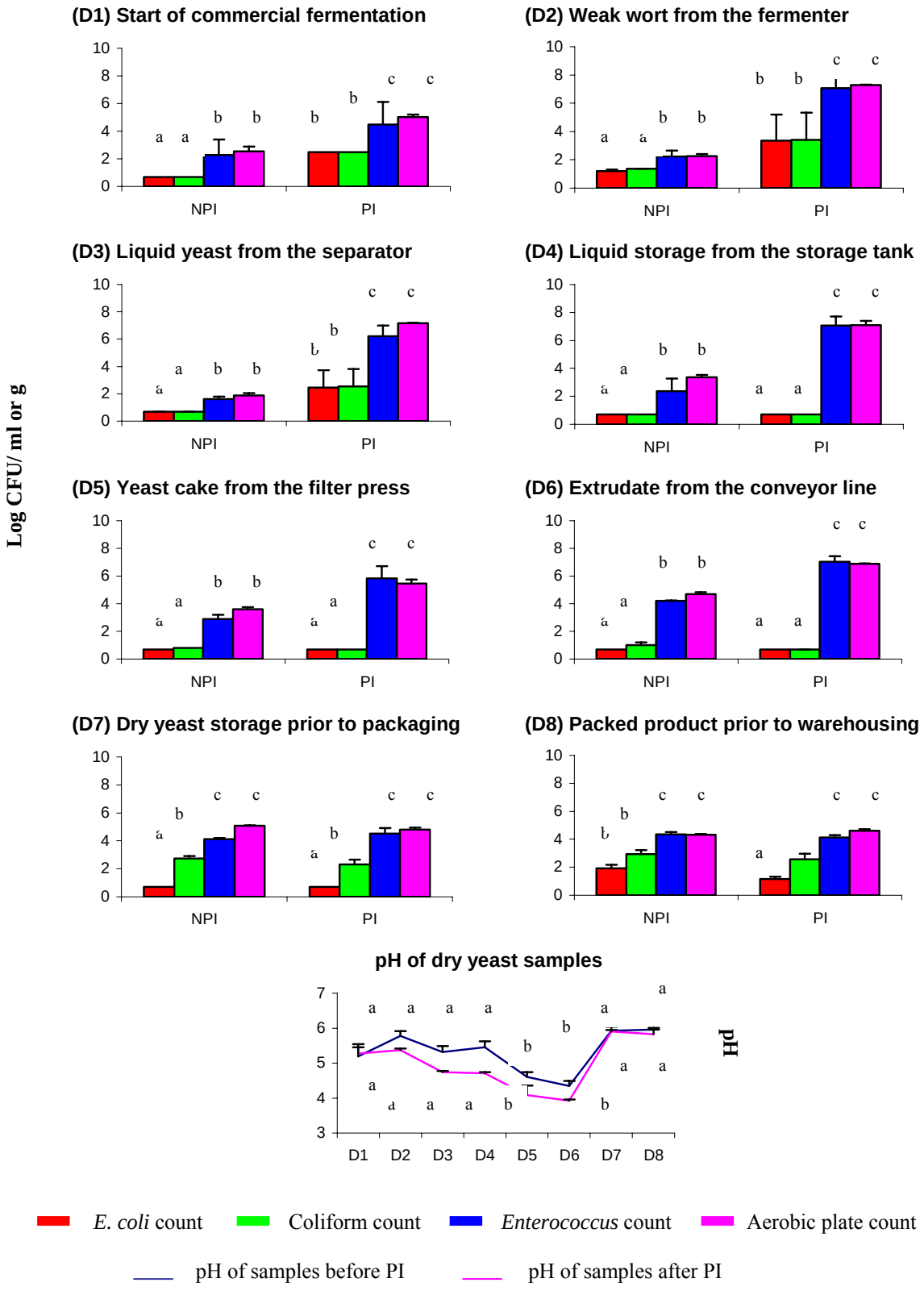


Figure 2.1.6 Mean *E. coli*, coliform, *Enterococcus* and aerobic plate counts (Log CFU/ ml or g), before and after preliminary incubation at 37°C for 24 h, over three replicate surveys for dry yeast production (Lower detection limit = 0.7 log CFU/ ml or g). Mean pH over three replicate surveys is shown for dry yeast production. Mean bacterial counts followed by the same superscripts are not significantly different (P > 0.05).

Table 2.1.3 Mean bacterial numbers for finished cream, compressed and dry yeast product samples taken during three independent replicate surveys of a commercial yeast manufacturing process

Finished product	Bacterial count (log CFU/ ml or g finished product)							
	***E. coli* count**		**Coliform count**		***Enterococcus* count**		**Aerobic plate count**	
	Non-PI[a]	**PI[b]**	**Non-PI**	**PI**	**Non-PI**	**PI**	**Non-PI**	**PI**
Dry yeast	1.93 ± 0.25[c]	1.17 ± 0.17	2.95 ± 0.26	2.56 ± 0.41	4.34 ± 0.19	4.12 ± 0.17	4.33 ± 0.28	4.60 ± 0.16
	(1.68 – 2.18)[d]	(1.00 – 1.34)	(2.69 – 3.21)	(2.15 – 2.97)	(4.15 – 4.53)	(3.95 – 4.29)	(4.05 – 4.61)	(4.44 – 4.76)
Cream yeast	1.00 ± 0.21	1.78 ± 0.77	1.20 ± 0.25	3.75 ± 0.93	4.13 ± 0.89	7.85 ± 0.30	5.20 ± 0.51	8.53 ± 0.41
	(0.79 – 1.21)	(1.01 – 2.55)	(0.95 – 1.45)	(2.82 – 4.68)	(3.24 – 5.02)	(7.55 – 8.15)	(4.69 – 5.71)	(8.12 – 8.94)
Compressed yeast	1.37 ± 0.47	1.13 ± 0.31	2.25 ± 0.34	2.30 ± 0.13	4.04 ± 0.68	7.76 ± 0.30	4.59 ± 0.78	7.95 ± 0.12
	(0.9 – 1.84)	(0.82 – 1.44)	(1.91 – 2.59)	(2.17 – 2.43)	(3.36 – 4.72)	(7.46 – 8.06)	(3.81 – 5.37)	(7.83 – 8.07)

[a] Samples analysed on day 0

[b] Corresponding samples analysed after preliminary incubation at 37^{o}C for 24 h

[c] Mean ± standard deviation

[d] Range

CHAPTER 2. 2

THE BACTERIAL ECOLOGY OF COMMERCIALLY MANUFACTURED YEAST DURING PROCESSING

ABSTRACT

Bacterial populations associated with the production of cream, compressed and dry yeast were characterized, before and after preliminary incubation (PI) of samples at 37°C for 24h. Characterization of 496 bacterial isolates, 244 from non-PI samples and 252 from PI samples, indicated the predominance of Gram-positive genera, comprising 91 to 100% of the total population during seed, cream, compressed and dry yeast production. Using a simplified characterization key based on morphological examination, members of the LAB family, *Lactobacillus* and *Enterococcaceae* (*Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*), predominated in samples collectively comprising 81 to 100% of the total population of commercially manufactured yeast. *Lactobacillus* was the primary isolate recovered from seed yeast fermentation samples, with *Enterococcaceae* recovered after the washing and separation of yeast cream. During refrigerated storage, there was an increase in the diversity of the bacterial population, with Gram-negative rods and *Micrococcus* comprising 8.3% of the total population. The filtering of yeast cream into yeast cake initiated the dominance of *Enterococcaceae* (60 to 70%) over *Lactobacillus.* Drying and vacuum-packaging of yeast reduced the dominance of *Lactobacillus* by 50%, resulting in an increased proportion of *Enterococcaceae* and Gram-negative rods. The spore-forming bacterium, *Bacillus*, responsible for the rope-spoilage of bread, comprised 8.3% of the final dry yeast product population. The high proportion of *Lactobacillus* in Baker's yeast can contribute positively to the leavening, aroma and overall flavour of bread. However, large proportions of *Enterococcaceae* in Brewer's yeast, used as a raw material in brewing, could possibly result in the spoilage of alcoholic beverages.

INTRODUCTION

Commercially propagated yeast is not a monoculture (Reed and Nagodawithana, 1991; Enfors, 2001) but frequently contains other associated microbial populations, predominantly bacteria, moulds and occasionally wild yeasts (Reed and Nagodawithana, 1991; Tessendorf *et al*., 1991). Processing, storage and subsequent handling exert stress on microorganisms (Hartman *et al.*, 1992), factors which could cause changes in the bacterial populations associated with cream, compressed and dry yeast production.

Commercial yeast contains a wide variety of freely available nutrients in the form of water (70%), proteins (15%), carbohydrates (10.5%), minerals (3%) and fats (1.5%), providing an ideal environment for the growth of contaminating microorganisms. Previous studies have found bacterial counts from finished compressed yeast product generally fall between 10^4 and 10^8 bacteria per gram (Reed and Nagodawithana, 1991; Viljoen and Lues, 1993). These bacterial contaminants include predominantly Gram-positive genera of the lactic acid bacteria (LAB) family (Reed and Nagodawithana, 1991; Lues, 1992), *Bacillus* spores (Bailey and von Holy, 1993; Viljoen and von Holy, 1997), and occasionally Gram-negative coliforms and *E. coli* (Reed and Nagodawithana, 1991; O'Brien *et al*., 2004a).

The concept of the LAB as a group of organisms developed in the beginning of the 1900's, and to date includes species of the genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissela* (Dykes *et al.*, 1993; Stiles and Holzapfel, 1997). LAB are Gram-positive, non-motile, non-spore forming, rod- and coccus-shaped bacteria that ferment carbohydrates into lactic acid (Stiles and Holzapfel, 1997). Despite their contribution to the spoilage of many foods, especially processed meats (Franz *et al*., 1991; Franz and von Holy, 1996; Franz *et al*., 2003), LAB have also been acknowledged for their use and beneficial role as starter cultures in the fermentation of traditional beverages (Haggblade and Holzapfel, 1989), yoghurt (Birollo *et al.*, 2001), cheese (Giraffa *et al.*, 1997) and fermented sausages (Franz *et al*., 2003). Due to their ability to reduce pH by the production of lactic acid, they are also recognized for their positive role in the preservation of some foods (Stiles and Holzapfel, 1997).

Commercial yeast products have typically low acid pH between 5 and 6, favourable for the growth and proliferation of members of the LAB family, which are capable of growth at pH values as low as 3.5, and are capable of enhancing their competitive abilities by producing lactic acid which further reduces pH (Reed and Nagodawithana, 1991). Thus, resulting in the well documented predominant LAB populations that reside within commercially manufactured yeast. Baker's yeast has been found to contain members of the LAB family, including *Leuconostoc*, *Lactobacillus* (Reed and Nagodawithana, 1991), *Pediococcus*, *Lactococcus* (Lues, 1992; Viljoen and Lues, 1993) and *Enterococcus* species (O'Brien *et al*., 2004a).

The assumption that all contaminating microbial genera found in commercial yeast are inhibitory to bread production is unfounded, since some species play an important role in taste and texture development (Clayton, 1973). LAB, in low numbers, can contribute positively to the role of yeast in baking, by increasing carbon dioxide production (Lonner and Preve-Akeson, 1989) as well as their ability in enhancing sensory properties by contributing to the overall aroma, flavour and texture of bread (Oura *et al*., 1982; Suigihara, 1985; Lonner and Preve-Akeson, 1989; Martinez-Anaya *et al*., 1990; Tessendorf, 1991). In addition, the presence of LAB in dough, increases the acidity of the environment by metabolizing carbohydrates into lactic acid, resulting in a decrease in the activity of unwanted contaminants such as *Bacillus*, moulds and Gram-negative coliforms (Reed and Nagodawithana, 1991; Tessendorf, 1991, Lues, 1992).

In brewing, the use of yeast contaminated with bacteria, can result in the spoilage and associated quality defects of beer. Gram-positive bacteria that are of significance to brewing include members of the LAB family, primarily *Lactobacillus* and *Pediococcus* (Reed and Nagodawithana, 1991). Lactobacilli are non-sporulating, catalase negative, rod-shaped bacteria, which are either homofermentative or heterofermentative (Prescott *et al*., 1996). They are readily isolated from beer, and constitute the predominant spoilage organisms found in the beer industry (Reed and Nagodawithana, 1991). In addition to the lowering of the pH of beer through the production of lactic acid, lactobacilli also produce diacetyl, often responsible for the "buttery" flavour and "silky" turbidity of spoilt beer (Reed and Nagodawithana, 1991).

In addition to lactic acid producing bacteria, endospore-forming bacteria belonging to the genus *Bacillus* and certain *Micrococcus* species have also reportedly caused problems in breweries (Reed and Nagodawithana, 1991). *Micrococcus kristinae* is relatively acid tolerant and has reportedly been responsible for beer spoilage (Reed and Nagodawithana, 1991). Some Gram-negative bacteria, including members of the family *Enterobacteriaceae*, have also been responsible for the spoilage of beer (Reed and Nagodawithana, 1991).

The presence of bacteria in commercially manufactured yeast, used as a raw material in baking or brewing, can therefore introduce a variety of spoilage organisms into bread production processes and breweries. The bacterial ecology of commercially manufactured yeast subsequent to initial contamination is influenced by a number of factors including temperature, pH and processing stages. Previous studies (Tessendorf, 1991; Viljoen and Lues, 1993) have focused on bacterial populations associated with finished commercial yeast products only. It is well documented that not all bacteria flourish under the same environmental conditions, thus only certain groups of bacteria are capable of contaminating commercial yeast in its different stages of production. This study therefore aimed to identify the bacterial populations associated with the commercial manufacture of yeast during the different stages of production and consequently the final populations that reside within the finished cream, compressed and dry yeast products.

MATERIALS AND METHODS

Bacterial populations were sampled during three replicate surveys of a commercial yeast factory over a period of six months (July to December 2003). In total 24 yeast samples were collected for each of the replicate surveys for seed (7), cream (1), compressed (8) and dry (8) yeast production (Chapter 2.1).

In order to determine the effect of increased temperature on the predominance of certain bacterial genera, bacterial populations were obtained before (non-PI) and after preliminary incubation (PI) of yeast samples at 37°C for 24 h. Two predominant colonies (where possible) were isolated from duplicate WL Nutrient agar (Oxoid, Basingstoke, UK) + 1% cyclohexamide (0.004g/ litre) (Scharlau, Barcelona, Spain) total aerobic plate count plates, using plates of highest dilutions

showing growth (von Holy and Holzapfel, 1988). In this way, a total of 496 isolates were obtained, 244 from non-PI samples and 252 from PI samples. The bacterial isolates were purified by standard methods (von Holy and Holzapfel, 1988) and characterized to genus level according to Gram and catalase reactions as well as morphology using the dichotomous key of Fisher *et al.* (1986) (Figure 2.2.1 and Figure 2.2.2). Gram-positive, catalase-negative cocci were grouped classified as *Enterococcaceae* and include members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus.* Furazolidone (FTO) agar was used to further differentiate *Micrococcus* spp. and *Staphylococcus* spp. (Sneath *et al*., 1981). Gram-negative bacterial isolates were not characterized further. An incubation temperature of 30°C was used in all cases.

RESULTS

The percentage distribution of bacterial populations isolated from aerobic plate count plates of non-PI and PI seed (Table 2.2.1; A and B), compressed and cream (Table 2.2.2; A and B) and dry (Table 2.2.3; A and B) yeast are shown.

(a) Characterization of bacterial populations from seed yeast production samples

No isolates were obtained for seed yeast samples (S1 to S3) taken prior to the end of the Mother fermentation (MF), as aerobic plate counts (APC) were below the lower detection limit (< 0.7 log CFU/ ml) (Chapter 2.1). Bacterial populations isolated from seed yeast samples (numbering 88), before and after PI, in this study were dominated by Gram-positive genera (*Lactobacillus* and *Enterococcaceae*) (100%; Table 2.2.1; A and B). *Lactobacillus* was the primary bacterium isolated during seed yeast production which dominated over *Enterococcaceae* (later introduced during washing and separation) before (66.7 to 100%) and after (50 to 66.7%) PI of samples (Table 2.2.1; A and B). Overall, however, the PI procedure decreased the dominance of *Lactobacillus* in seed yeast samples from 72.5 to 63.6%, with an increase in the frequency of *Enterococcaceae* from 27.5 to 36.4% (Table 2.2.1; B).

(b) Characterization of bacterial populations from cream and compressed yeast production samples

For both, non-PI and PI, isolates taken during cream and compressed yeast production (numbering 216), Gram-positive groups (*Lactobacillus*, *Enterococcaceae* and *Micrococcus*) collectively comprised 97.2% of isolates, and Gram-negative rods 2.8% of isolates (Table 2.2.2; A and B). Results show the dominance of *Lactobacillus* (50 to 66.7%) over *Enterococcaceae* during the early stages of cream and compressed yeast production following commercial fermentation with seed yeast (Table 2.2.2, A, sample C1 to C4). However, the filtering of yeast cream into yeast cake initiated the dominance of *Enterococcaceae* over *Lactobacillus* (58.3 to 75%; Table 2.2.2, A, sample C6 to C9) during subsequent stages of compressed yeast processing. Yeast cream samples taken from the storage tank initiated the isolation of *Micrococcus* (8.3%) and Gram-negative rods (8.3%) in cream and compressed yeast samples (Table 2.2.2, A, sample C4). Finished cream yeast product contained 41.7% *Enterococcaceae*, 41.7% *Lactobacillus*, 8.3% *Micrococcus* and 8.3% Gram-negative rods (Table 2.2.2, A, sample C5). Finished compressed yeast product contained 66.7% *Enterococcus*, 33.3% *Lactobacillus* and 8.3% Gram-negative rods (Table 2.2.2, sample C9). In general, PI increased the percentage of *Enterococcaceae* in populations obtained from cream and compressed yeast samples.

(c) Characterization of bacterial populations from dry yeast production samples

Isolates collected during the production of dry yeast (numbering 192), were also predominantly Gram-positive (*Lactobacillus*, *Enterococcaceae*, *Micrococcus* and *Bacillus*) for both non-PI (91%) and PI (95%) samples. Gram-negative rods comprised 10% and 5% of the overall bacterial population isolated during dry yeast production for non-PI and PI samples, respectively (Table 2.2.3; A and B). Similar to cream and compressed yeast production samples, *Enterococcaceae* dominated over *Lactobacillus* in the majority of samples taken during the production of dry yeast, and especially after PI (Table 2.2.3; A and B). *Micrococcus* and Gram-negative rods were first isolated from yeast samples taken upstream of the process, during commercial fermentation and after separation of the yeast cream, respectively (Table 2.2.3; A, sample D1 and D3). The frequency of *Micrococcus* in dry yeast samples was higher than cream and compressed yeast

samples, comprising 8% of the bacterial population throughout the production of dry yeast prior to vacuum-packaging. Vacuum-packaged dry yeast was the only yeast sample to contain *Bacillus* (1%; Table 2.2.3; A, sample D8). Finished dry yeast product contained 58% *Enterococcaceae*, 33% *Lactobacillus*, 9% Gram-negative rods and 1% *Bacillus* (Table 2.2.3; A, sample D8).

DISCUSSION

LAB (*Enterococcaceae* and *Lactobacillus*) dominated bacterial populations associated with cream, compressed and dry yeast manufacturing, with proportions ranging from 33.3 to 72.5% and 27.5 to 56.3%, respectively. This was expected since previous studies have found LAB, namely *Lactobacillus* to dominate bacterial populations associated with Baker's compressed yeast (Seiler, 1975; Wood and Hodge, 1985; Tessendorf *et al*., 1991; Viljoen and Lues, 1993). In addition, *Enterococcaceae* have previously been isolated from raw materials, including Baker's compressed yeast, used in the commercial manufacture of bread (Viljoen and von Holy, 1997, O'Brien *et al*., 2004a).

Although lactobacilli are recognized for their positive role in reducing the growth of Gram-negative bacteria in commercial yeast and dough, as well as in their favourable contribution to the leavening, aroma and taste of bread, lactobacilli are, however, major contributors to the spoilage of beer and wine fermented from commercial yeast. Lactobacilli are capable of multiplying at low storage temperatures (Sugihara 1985; Wood and Hodge, 1985), such as those used in the storage of cream and compressed yeast products (*ca*. 4°C), thus are capable of multiplying to high levels during cold storage. According to the South African Bureau of Standards (SABS), guidelines restrict the number of *Lactobacillus* in Baker's and Brewer's yeast to 5.7 log CFU/ g (SABS 1642, 1995; SABS 1643, 1995). In this study, no counts were determined for LAB, specifically *Lactobacillus*, during surveys of the commercial yeast factory (Chapter 2.2). Thus, future studies should determine LAB counts associated with commercial yeast, since results from this study show *Lactobacillus* constitutes a large portion of the bacterial population associated with the commercial manufacture of cream, compressed and dry yeast.

During the production of seed yeast, meant as an inoculum for commercial fermentations, Gram-positive rods, namely lactobacilli, dominated over *Enterococcaceae* in all seed yeast production line samples, before and after preliminary incubation. During the production of seed yeast, the pH is maintained within in the acidic range (*ca*. 4 to 5) with the addition of phosphoric acid, as a means of reducing the growth and/ or proliferation of bacterial contaminants. The ability of LAB, especially *Lactobacillus*, to survive and grow at pH values as low as 3.5 (Reed and Nagodawithana, 1991), may explain their dominance during the early stages of cream, compressed and yeast production, compared to upstream processing stages, where the pH increases to *ca.* 5 to 6, thus becoming more favourable for the contamination and growth of other LAB such as *Enterococcaceae*. For example, the liquid yeast taken after the separation of yeast cream initiated the dominance of *Enterococcaceae* over *Lactobacillus*, with proportions increasing from 30 to 40% (non-PI) and 50 to 100% (PI).

Gram-negative bacteria were isolated during the production of cream, compressed and dry yeast, but at relatively low proportions (*ca*. 8% of total population). The presence of Gram-negative bacteria in yeast samples taken after the cold storage of yeast cream, during the production of cream and compressed yeast, correlated well with our previous findings that the cold storage tank was the initial site of contamination of yeast product with Gram-negative coliforms (Chapter 2.1). The low proportions of Gram-negative bacteria in this study, is thought to be due to high numbers of competing bacteria, for example LAB (Viljoen and Lues, 1993). Gram-negative bacteria are unable to dominate the spoilage ecology of commercial yeast (Reed and Nagodawithana, 1991). Their growth is largely inhibited by microbially produced lactic acid and the consequent pH decreases which occur during the growth of LAB (Franz, 1993). This phenomenon has previously been reported in dough during the commercial production of bread (Lues, 1992).

The vacuum-packaging of dry yeast directly influences the atmosphere to which bacteria are exposed. Vacuum-packaging decreases the amount of oxygen available for the proliferation of contaminants. These conditions, however, reportedly favour the growth of microaerophilic and/ or anaerobic microorganisms such as LAB (Dykes *et al*., 1991; Dykes *et al.*, 1993). In this study, members of the LAB family, *Enterococcaceae* and *Lactobacillus* collectively comprised 83.3%

of isolates from vacuum-packaged dry yeast product. Gram-positive *Bacillus* (*B.*) spp. which are well known for the formation of 'rope' in bakery products (Kirschner and von Holy, 1989) were only isolated from finished dry yeast product in this study, comprising 8% of the bacterial population, before and after preliminary incubation of samples at 37°C for 24h. This finding correlated with previous findings, which found dry yeast to be the only finished product containing spore-forming bacteria, namely *B. megaterium*, *B. licheniformis* and *B. subtilis* (Chapter 2.2).

Previous studies of South African bakery, have found *Micrococcus* species present in dough comprising 5% of the total bacterial population, but not in Baker's compressed yeast (Viljoen and Lues, 1993). However, results from this study found *Micrococcus* was readily isolated during the commercial production of cream, compressed and dry yeast comprising 8% of the population for most of the yeast samples, subsequently residing within finished cream, compressed and dry yeast product. The presence of *Micrococcus* in finished commercial yeast products can result in the spoilage of beer and wine, if used as a raw material in alcoholic fermentations (Reed and Nagodawithana, 1991), however, micrococci have little pathogenic potential, other than the occasional link to opportunistic infections (Sleigh and Timbury, 1998).

In a previous study, a preliminary incubation (PI) procedure was efficient in increasing bacterial counts associated with commercially manufactured yeast (O'Brien *et al.*, 2004a). Results from this study found bacterial populations isolated after PI were similar in composition to the populations obtained from non-PI yeast samples. However, populations were found in different ratios after pre-incubation, with an increase in the predominance of *Enterococcaceae*. Our findings showed that preliminary incubation is a valid method for increasing bacterial counts associated with commercially manufactured yeast and furthermore showed the possible effects of elevated temperature during processing, storage and distribution, which could result in the increased dominance of *Enterococcaceae* over the more 'favourable' contaminant *Lactobacillus*.

CONCLUSION

Several studies (Reed and Nagodawithana, 1991; Tessendorf *et al*., 1991; Viljoen and Lues, 1993; Enfors, 2001; O'Brien *et al*., 2004a) have provided evidence that Baker's and Brewer's yeast can contain a wide variety of bacteria. Based on the results obtained in this study it was concluded that LAB (*Enterococcaceae* and *Lactobacillus*), are present throughout the production of commercial yeast and constitute the predominant population in finished cream, compressed and dry yeast products. The high proportions of LAB, indicated the ability of these bacteria to survive and grow in the acidic pH of the seed and commercial fermentations and further processing into cream, compressed and dry yeast products. Gram-negative populations were not commonly recovered from yeast samples or finished yeast products in this study, suggesting the acid producing bacteria (*Enterococcaceae* and *Lactobacillus*) belonging to the predominantly Gram-positive population in commercially manufactured yeast may inhibit their growth. This is advantageous since Gram-negative bacteria can inhibit gas production by the yeast and cause fruity off-flavours in bread. The high proportion of *Enterococcaceae*, including enterococci, in Brewer's yeast used in the alcoholic fermentation of beer and wine is unfavorable, since it is considered an emerging pathogen, capable of causing disease. This is also true for *Bacillus* and Gram-negative rods isolated in this study, which may, not only result in the spoilage and quality defects of wine and beer, but may also question its safety for consumption.

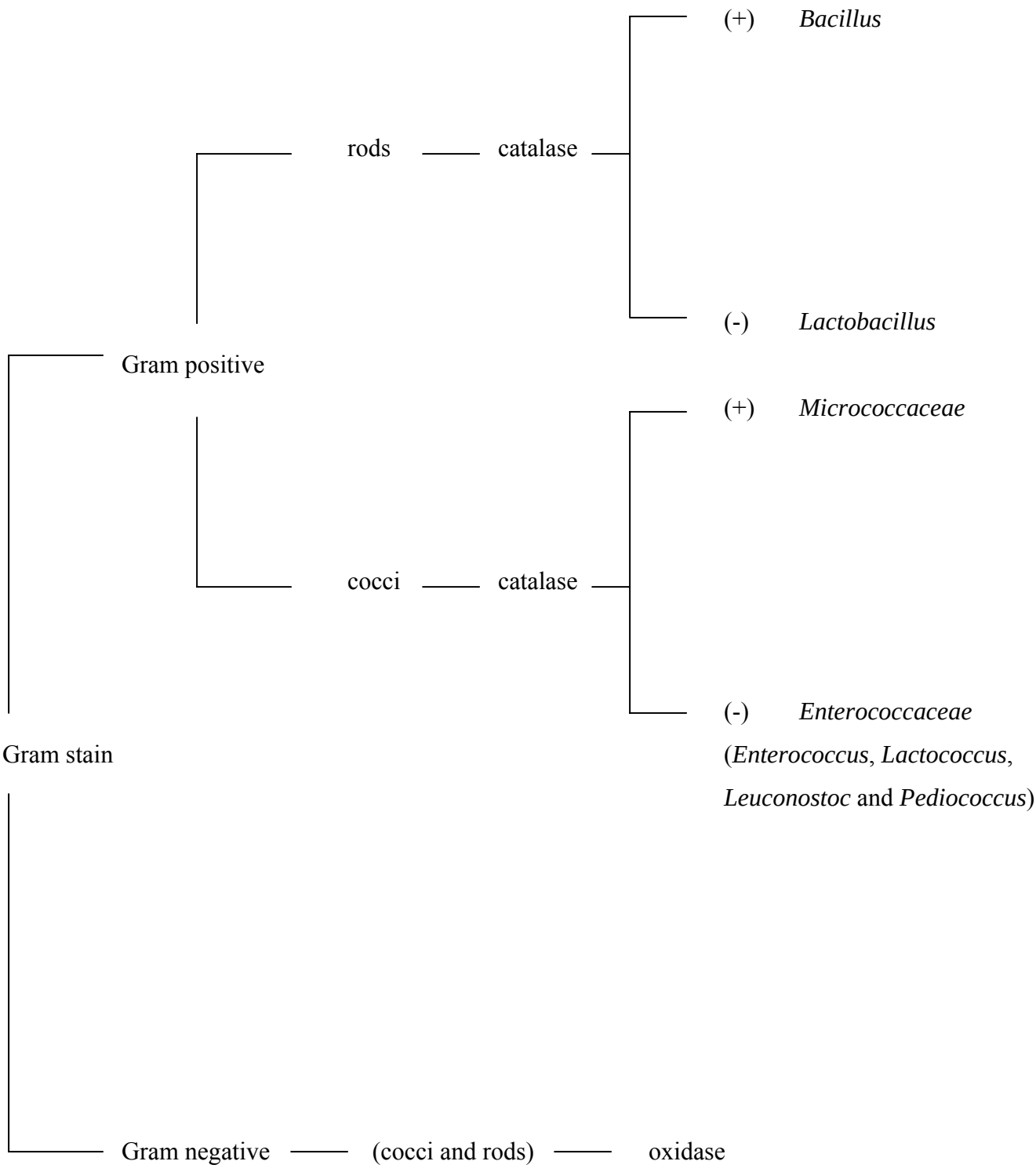


Figure 2.2.1 Modified characterization key (after Fischer *et al.*, 1986) for colonies isolated from aerobic plate count (APC) plates of WL Nutrient agar plus 1% cyclohexamide, for seed, cream, compressed and dry yeast samples.

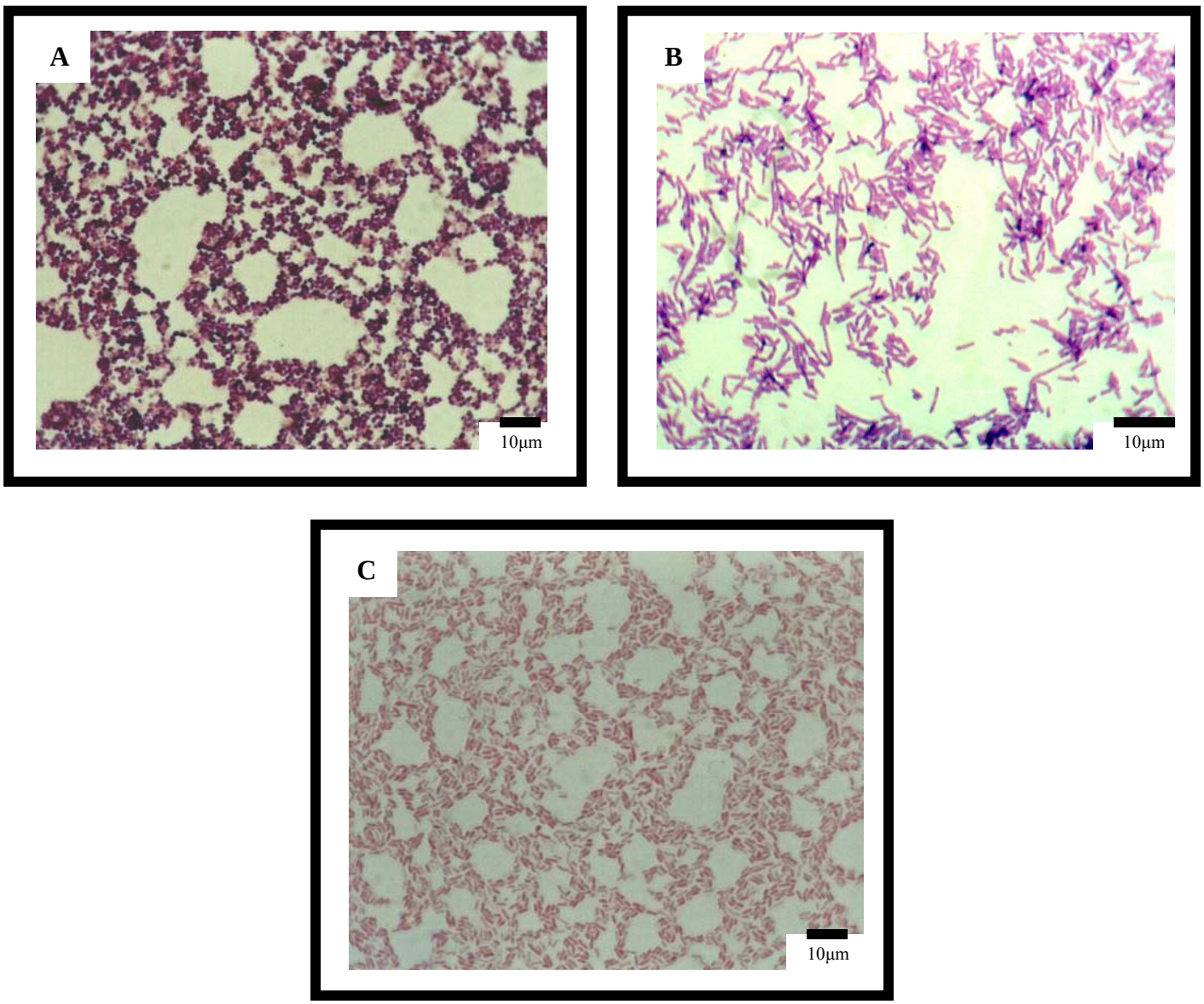


Figure 2.2.2 Light micrographs (x 1000 oil) of typical Gram-positive (purple) cocci **(A)**, Gram-positive rods **(B)** and Gram-negative (red) rods **(C)** isolated from yeast samples

Table 2.2.1 Percentage distribution of 88 isolates from aerobic plate counts of seed yeast samples before **(A)** and after **(B)** preliminary incubation at 37°C for 24h

	Sample	Description (No. of isolates)	% *Lactobacillus*	% *Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram-negative
A	**S4**	End of MF[b] before separation (4)	100	0	0	0	0
	S5	Seed cream during separation (12)	67	33	0	0	0
	S6	Separated cream from the liquid yeast vessel (before acid treatment) (12)	75	25	0	0	0
	S7	Separated cream from the liquid yeast vessel (after acid treatment) (12)	67	33	0	0	0
		TOTAL = 40	**73**	**27**	**0**	**0**	**0**
		% Gram-positive	**100 (73 % rods and 27 % cocci)**				
B	**S4**	End of MF before separation (12)	50	50	0	0	0
	S5	Seed cream during separation (12)	67	33	0	0	0
	S6	Separated cream from the liquid yeast vessel (before acid treatment) (12)	67	33	0	0	0
	S7	Separated cream from the liquid yeast vessel (after acid treatment) (12)	67	33	0	0	0
		TOTAL = 48	**64**	**36**	**0**	**0**	**0**
		% Gram-positive	**100 (64 % rods and 36 % cocci)**				

[a] *Enterococcaceae* (collectively *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)
[b] Mother fermentation

Table 2.2.2 Percentage distribution of 216 isolates from aerobic plate counts of cream and compressed yeast samples before **(A)** and after **(B)** preliminary incubation at 37°C for 24h

	Sample	Description (No. of isolates)	% *Lactobacillus*	% *Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram-negative
A	**C1**	Start of commercial fermentation (12)	58	42	0	0	0
	C2	Weak wort from the fermenter (12)	67	33	0	0	0
	C3	Liquid yeast from the separator (12)	58	42	0	0	0
	C4	Liquid storage from the storage tank (12)	50	33	8	0	8
	C5	Finished cream yeast product (12)	42	42	8	0	8
	C6	Yeast cake from the filter press (12)	42	58	0	0	0
	C7	Yeast cake from hopper (12)	17	75	8	0	0
	C8	Block yeast from the packing line (12)	33	67	0	0	0
	C9	Wrapped compressed yeast product prior to cold storage (12)	25	67	0	0	8
		TOTAL = 108	**44**	**50**	**3**	**0**	**3**
		% Gram-positive		**97 (55 % cocci and 45 % rods)**			
B	**C1**	Start of commercial fermentation (12)	42	58	0	0	0
	C2	Weak wort from the fermenter (12)	50	50	0	0	0
	C3	Liquid yeast from the separator (12)	0	100	0	0	0
	C4	Liquid storage from the storage tank (12)	58	42	0	0	0
	C5	Finished cream yeast product (12)	33	50	8	0	8
	C6	Yeast cake from the filter press (12)	33	67	0	0	0
	C7	Yeast cake from hopper (12)	59	33	8	0	8
	C8	Block yeast from the packing line (12)	50	42	0	0	0
	C9	Wrapped compressed yeast product prior to cold storage (12)	33	50	8	0	8
		TOTAL = 108	**40**	**54**	**3**	**0**	**3**
		% Gram-positive		**97 (59 % cocci and 41 % rods)**			

[a] *Enterococcaceae* (collectively *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)

[b] Mother fermentation

Table 2.2.3 Percentage distribution of 192 isolates from aerobic plate counts of dry yeast samples before **(A)** and after **(B)** preliminary incubation at 37°C for 24h

	Sample	Description (No. of isolates)	% *Lactobacillus*	% *Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram-negative
A	**D1**	Start of commercial fermentation (12)	50	42	8	0	0
	D2	Weak wort from the fermenter (12)	58	33	8	0	0
	D3	Liquid yeast from the separator (12)	25	33	8	0	33
	D4	Liquid storage from the storage tank (12)	25	42	8	0	25
	D5	Yeast cake from the filter press (12)	25	67	8	0	0
	D6	Extrudate from the conveyor line prior to drying (12)	50	42	8	0	0
	D7	Dry yeast storage prior to packaging (12)	25	42	17	0	17
	D8	Packed dry yeast product prior to warehousing (12)	33.3	58	0	8	0
		TOTAL = 96	**37**	**45**	**8**	**1**	**9**
		% Gram-positive		**91 (59 % cocci and 41 % rods)**			
B	**D1**	Start of commercial fermentation (12)	50	50	0	0	0
	D2	Weak wort from the fermenter (12)	42	58	0	0	0
	D3	Liquid yeast from the separator (12)	0	75	8	0	17
	D4	Liquid storage from the storage tank (12)	58	25	8	0	8
	D5	Yeast cake from the filter press (12)	50	50	0	0	0
	D6	Extrudate from the conveyor line prior to drying (12)	50	33	8	0	8
	D7	Dry yeast storage prior to packaging (12)	8	92	0	0	0
	D8	Packed dry yeast product prior to warehousing (12)	8	67	8	8	8
		TOTAL = 96	**33**	**56**	**4**	**1**	**5**
		% Gram-positive		**95 (64 % cocci and 36 % rods)**			

[a] *Enterococcaceae* (collectively *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)

[b] Mother fermentation

CHAPTER 2.3

THE PRESENCE OF *LISTERIA MONOCYTOGENES* AND OTHER FOODBORNE BACTERIAL PATHOGENS IN COMMERCIALLY MANUFACTURED YEAST

ABSTRACT

The occurrence of *Listeria*, *Salmonella*, *Staphylococcus* (*S*.) *aureus* and bacterial spores in cream, compressed and dry yeast product was studied. Finished yeast product samples were collected on three occasions over 6 months (July to December 2003) and were tested before (non-PI) and after preliminary incubation (PI) of samples at 37°C for 24 h. In this study, no *Salmonella* or *S*. *aureus* were found in any of the three finished yeast product samples, before or after PI. All cream yeast samples tested negative for the presence of bacterial spores and *Listeria*, whilst all compressed yeast samples tested negative for the presence of bacterial spores. Bacterial spores were found in 33% (non-PI) and 67% (PI) of dry yeast samples, *ca*. 3 and 1 log CFU/ g, respectively. Spore-forming bacteria isolated from dry yeast samples were identified as *Bacillus* (*B.*) *megaterium*, *B. licheniformis* and *B*. *subtilis*, species commonly associated with the rope-spoilage of commercially manufactured bread. For dry yeast, 67% (non-PI) and 100% (PI) of samples tested positive for *L. monocytogenes* compared with 33% (non-PI) and 67% (PI) of compressed yeast samples. It is thought that the processing environment may be serving as a source of *Listeria* contamination of finished compressed and dry yeast product. We also concluded that regular *L. monocytogenes* testing of finished compressed and dry yeast product samples should be performed since recommended guidelines require the absence of *L. monocytogenes* in 25 g commercial yeast product for use in baking and/ or brewing. This will also allow for the efficient monitoring of possible persistent *L. monocytogenes* contamination in the commercial yeast factory and its sources.

INTRODUCTION

The presence of bacterial pathogens in commercial yeast is not well documented and is of concern since "edible" commercial yeast (SABS 1642, 1995; and SABS 1643, 1995) is used as a raw material in a variety of commercial processes. For example, commercial yeast is used as a leavening agent in the commercial production of bread, as well as in the fermentation of alcoholic beverages. In addition, Baker's and Brewer's yeast are also used in the production of yeast extracts, by a controlled process of autolysis (Peppler, 1982; Champagne *et al*., 1999), which are commonly used as flavour enhancers or flavouring ingredients in the food industry (Reed and Nagodawithana, 1991).

Even though the high temperatures used in the commercial production of bread (*ca*. 250°C) (Bailey and von Holy, 1993), generally 'bake off' any bacterial contaminants and/ or potentially pathogenic bacteria which may be present, the presence of pathogens is still of concern, not only from a quality and hygiene point of view, but more importantly from a safety aspect. The use of Baker's yeast containing *L. monocytogenes* may act as a vehicle of entry into bakeries, possibly resulting in the cross-contamination of baked goods. This is also true for the use of commercial yeast (normally dry Brewer's yeast) used in the fermentation of alcoholic beverages such as beer, wine and sorghum beer.

Sorghum beer is a soured fermented drink, sold in a state of active yeast fermentation, indigenous to sub-Saharan Africa (Pattison *et al*., 1998). The safety of sorghum beer fermented from commercial yeast containing pathogens may be questionable since sorghum beer has a relatively low alcohol content (less than 3%), is not pasteurized (Novellie, 1963) and is commonly stored at room temperature at the point of sale (Haggblade and Holzapfel, 1989). All of these factors would not prevent the survival and/ or outgrowth of pathogens. In addition, the temperature *ca*. 20°C (Pattison *et al*., 1998), at which the yeast is added during the production of sorghum beer is not high enough to kill pathogenic bacteria, such as *Listeria* (*L*.) *monocytogenes* belonging to the lactic acid bacteria (LAB) family, which are reportedly common contaminants and contributors to the spoilage of sorghum beer fermentations (Haggblade and Holzapfel, 1989).

Listeriae are psychrotrophic, Gram-positive, non-sporing often motile rods, and facultative anaerobes (Shapton and Shapton, 1991; Prescott *et al*., 1996), capable of growth at a range of temperatures, from 0 to 45°C (Shapton and Shapton, 1991; Gall *et al*., 2004), and pH values, from 5 and 9.5 (Shapton and Shapton, 1991). The genus currently contains six species: *L. monocytogenes*, *L. ivanovii*, *L. welshimeri*, *L. innocua*, *L. seeligeri* and *L. grayi*, of which *L. monocytogenes* and *L. ivanovii* are pathogenic to humans and animals, respectively (Vazquez-Boland *et al*., 2001). *L. monocytogenes* is now firmly established as an important foodborne pathogen (Arihara *et al*., 1991) due to its potential in causing serious illness, and even death, in susceptible individuals.

L. monocytogenes is widespread in the environment, and has been found in soil, water, sewage, decaying vegetation, and can also be readily isolated from food processing plants (Jay 1998; Gall *et al*., 2004). *L. monocytogenes* has been isolated from raw materials, the environment, final products (Lundén, 2004) including fermented sausages, raw milk, raw vegetables and raw meat (Shapton and Shapton, 1991) as well as from condensate-forming equipment surfaces in food processing plants (Hassan *et al*., 2004). Due to the ubiquitous nature of *L. monocytogenes*, it is possible for constant re-introduction of the organism into a processing plant environment (Gall *et al*., 2004). At 4°C, listeriae are capable of slow growth, with a generation time of 18 – 30 hours (Shapton and Shapton, 1991), thus its presence in compressed yeast, together with its capability of growth at low pH of 5, during production and subsequent storage at 4°C is of concern.

Staphylococci are Gram-positive, non-motile cocci, and facultative anaerobes (Prescott *et al*., 1996). The growth temperature of staphylococci ranges from 6.5°C to 46°C with optimum of 37°C (Shapton and Shapton, 1991). The growth pH of staphylococci ranges from 4.2 to 9.3 with optimum of 7 (Prescott *et al*., 1996). Staphylococci are a common part of the human microflora (Jay, 1998), carried in the upper respiratory tract, in the mucous membranes of the nose, and on skin surfaces (Hobbs, 1974; Troller, 1983). They are primarily introduced into food processing environments by direct or indirect human contact (Prescott *et al.*, 1996), and have been proposed indicators of hand-to-food contact (Ingham and Lau, 2003). However, endemic *S. aureus* has been known to establish itself on food processing equipment contaminating passing product (Shapton and Shapton, 1991). Contamination with *S. aureus* is possible at the late stages of

compressed and dry yeast production during filtration and packaging, since these are the only stages where the product may be physically handled. It is therefore important to ensure that employees who handle yeast or yeast-contact surfaces display good hygiene practices by not transferring staphylococci to the product.

The genus *Salmonella* consists of Gram-negative, non-spore forming rod-shaped organisms that grow aerobically or anaerobically (Troller, 1983). Salmonellae will grow over a pH range of 4.1 – 9 and a water activity (a_w) range greater than 0.95 (Shapton and Shapton, 1991). The most favorable temperature for growth is 37°C, however, growth in foods has occurred at temperatures as low as 5 - 7°C (Troller, 1983, Shapton and Shapton, 1991). Salmonellae are found almost everywhere in the environment, in the gastrointestinal tracts of humans, birds and insects and in water and soil (Prescott *et al*., 1996). Salmonellosis is the result of an infection of the gastrointestinal tract, brought about by the ingestion of an infectious dose of salmonellae (Troller, 1983) with symptoms characterized by diarrhea and vomiting (Hobbs, 1974). To date, there is no documentation on the presence of *Salmonella* in commercially manufactured yeast, or the probability of *Salmonella* contaminating its associated fermented products.

Previous studies (Collins *et al*., 1991; Bailey and von Holy, 1993; Viljoen and von Holy, 1997) have provided evidence that yeast can contain *Bacillus* spores, and that its use as a raw material in commercial bread production may result in rope-spoilage of bread resulting in financial losses to the South African baking industry (Kirschner and von Holy, 1989). Rope spoilage of bread results from heat resistant spores produced by *Bacillus* that survive baking and germinate in the bread after baking (Hoffman *et al*., 1973) and is characterized by a distinctive fruity odour and a soft, sticky crumb (Kirschner and von Holy, 1989; Bailey and von Holy, 1993).

Guidelines for commercially manufactured yeast require the absence of pathogenic organisms (Reed and Nagodawithana, 1991; SABS 1642, 1995; SABS 1643, 1995), which would include *Listeria*, *Salmonella* and *S. aureus* in finished commercial yeast product. US military criteria for compressed Baker's and dry Brewer's yeast restrict the number of rope-causing *Bacillus* spores to less than 2 log CFU/ ml or g (Shapton and Shapton, 1991). For the exportation of locally manufactured yeast, safety is non-negotiable in international markets. This study therefore

assessed a local yeast manufacturer for the presence of foodborne pathogens in finished cream, compressed and dry yeast products.

MATERIALS AND METHODS

(a) Sample description and collection

During three replicate surveys of a commercial yeast factory for sources of bacterial contaminants (Chapter 2.1), finished product samples for cream (3), compressed (3) and dry (3) yeast were evaluated for the presence of pathogenic bacteria, *L. monocytogenes*, *Salmonella*, *S. aureus*, as well as general bacterial spores. For each replicate survey, finished cream, compressed and dry yeast samples were aseptically sampled as described in Chapter 2.1.

(b) Sample preparation for pathogen analysis

All finished product samples were analysed for the presence of selected bacterial pathogens and general bacterial spores on the day of sampling and after preliminary incubation (PI) of samples at 37^{o}C for 24 h.

Detection of *Listeria monocytogenes*

For primary enrichment of *L. monocytogenes*, 25 ml (cream yeast) or 25 g (compressed and dry yeast) were mixed with 225ml Fraser ½ broth (Bio-rad, Marnes-La-Coquette, France) and incubated at 30°C for 24h. For secondary enrichment 0.1ml of the incubated Fraser ½ broth was inoculated into 10 ml Fraser 1 broth (Bio-rad) and incubated at 37°C for 48h. A loopful of this culture was then streaked onto Rapid' L. Mono agar (Bio-rad) plates and incubated at 37°C for 48 h (Figure 2.3.1). Blue colonies without yellow halos were indicative of the presumptive presence of *L. monocytogenes*. Isolates which were Gram-positive, catalase-positive rods were further characterized using the BBL CRYSTAL™ Gram-Positive Identification system (Becton, Dickinson and Company, USA) (Figure 2.3.2).

Detection of *Salmonella*

Pre-enrichment for *Salmonella* spp. was carried out by inoculating 225 ml buffered peptone water (Oxoid, Basingstoke, UK) with 25 ml (cream yeast) or 25 g (compressed and dry yeast) followed by incubation at 37°C for 18 h. Aliquots (0.1 ml) of the enriched culture in BPW was then inoculated into 10 ml Rappaport Vassiliadis Soya (RVS) broth (Bio-rad) and incubated at 41.5°C for 24 h. A loopful of the RVS broth was then streaked onto Xylose – Lysine Desoxycholate (XLD) agar (Bio-rad) plates and incubated at 37°C for 24h. Red colonies with black centres were indicative of the presumptive presence of *Salmonella* (Figure 2.3.3).

Staphylococcus aureus count

For cream yeast, 10 ml aliquots were aseptically sampled and homogenized for 2 minutes using a Colworth 400 Stomacher (Seward Medical, London, UK) before analysis. For compressed yeast, 10 g aliquots were aseptically sampled and homogenized in 90 ml 0.85% NaCl (Biolab, Midrand, South Africa) as described above. For dry yeast samples, 10 g aliquots were aseptically sampled and incubated in 90 ml 0.85% NaCl (Biolab) at 37^{o}C for 30 minutes, and homogenized as described above. The 30 minute incubation step facilitated the rehydration of yeast cells as well as the recovery of any contaminants present before analysis (O'Brien *et al.*, 2004a). Serial dilutions of the homogenates were prepared in 0.85% NaCl (Biolab). One ml of each dilution was added to duplicate 3M™ Petrifilm™ Rapid *S. aureus* count plates (3M, St. Paul, U.S.A) and incubated for 24 ± 2 h at 37°C, and a further 1h at 62°C. After incubation, a single Thermostable Nuclease reactive disc plates (3M) was aseptically added to each of the Petrifilm™ plates and further incubated for 1 hour at 37°C. Petrifilm™ plates showing between 30 and 300 (or the highest number if below 30) red or blue colonies surrounded by pink zones were counted (Figure 2.3.4).

Bacterial spore count

Homogenates of cream, compressed and dry yeast product samples were prepared as described above, using Tryptone soya broth (TSB) (Oxoid) as the primary diluent, instead of BPW.

Bacterial spore counts were obtained by heating 10 ml of the 10^0 (cream yeast) or 10^{-1} (compressed and dry yeast) dilutions at 80°C for 13 min in a thermostat-controlled water bath, followed by cooling on ice for 3 min and duplicate plating using Tryptone Soya Agar (TSA) (Oxoid) reinforced with 1.5% (w/ v) bacteriological agar (Biolab) (Mossel *et al*., 1995; Kunene *et al*., 1999). Plates showing between 30 and 300 (or the highest number if below 30) white and cream colonies were counted (Figure 2.3.5). Isolates which were Gram-positive, catalase positive rods were further characterized using the BBL CRYSTAL™ Gram-Positive Identification system (Becton).

RESULTS

(a) Bacterial pathogens and bacterial spores in finished cream, compressed and dry yeast products

On three sampling occasions, the incidence of selected pathogens (*Listeria*, *Salmonella*, *S. aureus*) and bacterial spores in finished cream, compressed and dry yeast products, before and after preliminary incubation of samples at 37°C for 24 h, is shown in Table 2.3.1. None of the pathogens or bacterial spores tested for in this study were detected in finished cream yeast product samples. In addition, *Salmonella* spp. and *S. aureus* were not detected in any of the finished compressed and dry yeast samples, even after preliminary incubation of samples at 37ºC for 24 h (Table 2.3.1). However, presumptive *L. monocytogenes* was detected in 33% (non-PI) and 67% (PI) of compressed yeast samples, and 67% (non-PI) and 100% (PI) of dry yeast samples (Table 2.3.1). Bacterial spores were detected in dry yeast samples only, approximating 33% (non-PI) and 67% (PI), *ca*. 3 and 1 log CFU/ g, respectively (Table 2.3.1).

(b) Confirmation of *L. monocytogenes* in compressed and dry yeast samples

Four presumptive *L. monocytogenes* colonies on Rapid' L. Mono agar plates from compressed and dry yeast samples were isolated and purified by two successive streaks on TSA (Oxoid) plates. In total, 4 (non-PI) and 8 (PI) isolates were obtained from compressed yeast samples, as well as 8 (non-PI) and 12 (PI) isolates from dry yeast product samples during three replicate

surveys. The purity of the selected isolates was confirmed with Gram stains and catalase tests. Presumptive *L. monocytogenes* isolates were identified as Gram-positive, catalase-positive rods. Confirmation of *L. monocytogenes* was obtained using BBL CRYSTAL™ Gram-Positive Identification systems (Becton) (Table 2.3.1).

(c) Confirmation of *Bacillus* spp. in dry yeast samples

Presumptive spore-formers from dry yeast samples, obtained by spore counts were obtained, were isolated and purified by two successive streaks on TSA (Oxoid) plates. In total, 4 (non-PI) and 4 (PI) isolates were obtained from dry yeast product samples during three replicate surveys. The purity of the selected isolates was confirmed with Gram stains and catalase tests. Presumptive *Bacillus* isolates were identified as Gram-positive, catalase positive rods. These isolates were then further identified using BBL CRYSTAL™ Gram-Positive Identification systems (Becton, Dickinson and Company). Positive identifications for *Bacillus* spp. using BBL CRYSTAL™ Gram-Positive Identification systems included: *B. megaterium*, *B. licheniformis* and *Bacillus subtilis*.

DISCUSSION

The commercial processing of cream, compressed and dry yeast product involves a number of processing steps. There is opportunity for product contamination with bacterial pathogens and bacterial spores, if present on processing equipment, in the surrounding processing environment or from personnel who may come into contact with the yeast during the final stages of production and packaging. In this study, cream yeast product appeared to be the superior of all three product types as it contained none of the pathogens nor bacterial spores tested for in this study. This may largely be due to the reduced number of processing steps used in production of cream yeast in comparison to compressed yeast production which includes: filtration, extrusion, cutting and packaging and dry yeast production which includes: filtration, extrusion, drying and packaging). Also, unlike compressed and dry yeast production, cream yeast production is generally a closed system of fermentation tanks, separators and storage tanks and is therefore less susceptible to the risks of environmental contamination with potentially pathogenic bacteria or bacterial spores.

Salmonella and *S. aureus* were not found in any of the commercial yeast products sampled in this study. *S. aureus* is normally associated with the skin, skin glands and mucous membranes of people (Prescott *et al*., 1996). The absence of *S. aureus* may be due to the fact that there is limited contact between personnel and product during manufacturing of cream, compressed and dry yeast. *Salmonella* is not a likely pathogen associated with commercially manufactured yeast since *Salmonella* are generally associated with raw and/ or unprocessed products such as beef, poultry and eggs (Prescott *et al*., 1996). In addition if present, Gram-negative pathogens are generally out-competed by commonly associated Gram-positive LAB in commercially manufactured yeast.

Results from this study, found that the preliminary incubation of samples for 24h at 37°C increased the detection of *L. monocytogenes* by 30% in compressed and dry yeast samples. This technique could be implemented, prior to standard enrichment procedures, for the detection of bacterial pathogens in food. To our knowledge, this is the first time temperature alone, as apposed to enrichment broth and temperature, has been used as a pre-enrichment step prior to standard enrichment steps for the increased detection of *Listeria* and/ or other foodborne pathogens.

During three replicate analyses, compressed yeast samples tested positive for the presence of *L. monocytogenes*. The presence of *L. monocytogenes* in compressed yeast was not unexpected, since *Listeria* is a member of the LAB family, which in turn are reportedly the most predominant contaminants of yeast products (Lues, 1992). In addition, *L. monocytogenes* is capable of growth at 4°C, at a pH of 5 (Shapton and Shapton, 1991), conditions very similar to the intrinsic properties of cold-stored compressed yeast. A study of the survival of *L. monocytogenes* in hot-smoked fish found *L. monocytogenes* capable of multiplying to high numbers at 5 and 10°C (Ingham and Lau, 2003), thus indicating, *L. monocytogenes*, could also be capable of growth in cold stored compressed yeast products. *Listeria* does not survive temperatures greater than 43°C (Collee *et al*., 1996), thus compressed yeast contaminated with *Listeria* and used for baking may not pose a health risk as baking temperatures are *ca.* 250°C (Bailey and von Holy, 1993). However, the presence of *L. monocytogenes* is unacceptable since guidelines for 'edible' compressed yeast require the absence of bacterial pathogens in 25g (SABS 1642, 1995).

In this study, dry yeast samples also tested positive for the presence of *L. monocytogenes*. Reportedly, some food-borne pathogens such as *L. monocytogenes* can grow in processed products, particularly if other competing food-spoilage bacteria have been inhibited by packaging techniques such as vacuum-packaging, resembling the type of packaging of dry yeast used in this study. With the use of preliminary incubation, results showed on all three occasions of sampling (over a period of 6 months) that dry yeast product was contaminated with presumptive *L. monocytogenes*. This suggests prolonged and/ or persistent contamination may exist in the commercial yeast factory investigated in this study. The eradication of persistent contamination has been shown to be difficult but not impossible with improved sanitation (Lundén, 2004).

Alcoholic fermentations, such as sorghum beer production, inoculated with yeast starter cultures, have a low pH which largely reduces growth of pathogenic bacteria (Pattison *et al.*, 1998; Kunene *et al.*, 1999); however, legislation still specifies "zero" tolerance for the presence of pathogens (SABS 1642, 1995; SABS 1643, 1995), such as *Listeria* in Brewer's yeast. The ability to meet "zero" tolerance is very difficult if not nearly impossible, thus it is important to identify the possible source(s) of *L. monocytogenes* and attempt to reduce or prevent their entry into compressed and dry yeast products.

When processed foods are contaminated with *L. monocytogenes*, the source is often the food processing plant environment (Hassan *et al*., 2004). *L. monocytogenes* has been shown to adhere to several food contact surfaces including stainless steel within 20 minutes of contact (Mafu *et al*., 1990), becoming more resistant to cleaning agents and disinfectants used in the sanitation of food processing plants (Lundén, 2004). *L. monocytogenes* may subsequently be transferred from insufficiently cleaned surfaces to passing product (Ingham and Lau, 2003).

A previous study of the contribution of raw materials as sources of bacterial spores in the commercial production of bread, found that Baker's yeast had the highest spore count (*ca*. 5 log CFU/ g) (Viljoen and von Holy, 1997). It was therefore, not unexpected that 33% of dry yeast samples tested in this study contained bacterial spores (*ca*. 3 log CFU/ g). In addition, several previous studies have shown that yeast used in baking can contain *Bacillus* spores (Collins *et al*., 1991; Bailey and von Holy, 1993; Viljoen and von Holy, 1997), which survive the baking

process and subsequently result in rope-spoilage of bread (Viljoen and von Holy, 1997; Kirschner and von Holy, 1989). In this study, BBL CRYSTAL™ Gram-Positive Identification systems were used for the identification of presumptive spore-forming *Bacillus* (*B.*), isolated from dry yeast samples. Isolates returned positive identifications for *B. megaterium*, *B. licheniformis* and *B. subtilis*. Major *Bacillus* species, previously reported rope - spoilage inducers in bread include *B. subtilis*, *B. licheniformis*, *B. megaterium* and *B. cereus* (Streuli and Staub, 1955; Collins *et al*., 1991). Rope-spoilage of bread by *Bacillus* spp. can cause economic losses to the baking industry (Viljoen and von Holy, 1997). We therefore suggest, the commercial yeast factory implement a bacterial spore specific environmental monitoring program, in the dry yeast manufacturing plant, in order to establish the possible source(s) of bacterial spores and their entry into the passing dry yeast product.

CONCLUSION

The risk associated with the use of commercial yeast containing bacterial pathogens appears minimal. However, the presence of these bacterial pathogens in finished product is undesirable in any processing plant. Due to the psychrotrophic nature of *L. monocytogenes*, the presence of this bacterial pathogen in Baker's compressed yeast has many safety implications in the refrigerated storage and distribution of this product. The presence of *Listeria* in finished compressed and dry yeast product is indicative of problems with hygiene and/ or the contamination status of the yeast manufacturing plant. Persistent *L. monocytogenes* contamination of compressed and dry yeast product may be the result of adherence of *L. monocytogenes* to processing contact surfaces, adaptation to disinfectants or insufficient cleaning of processing equipment used in the final stages of yeast production and packaging. "Wet cleaning" in plants with multiple processing lines has been shown to increase the chances of product contamination with *L. monocytogenes* (Lundén, 2004). This has been seen in the commercial production of compressed yeast in this study, where two adjacent production lines are run alternatively, and while one of the lines is operating the other is cleaned. Thus, in avoiding cross-contamination of exposed yeast product with *L. monocytogenes*, we suggest cleaning should not be started if another line is still operating.

Improved cleaning and sanitation procedures for *Listeria* should focus on the most likely sources of direct yeast product contamination, such as equipment contact surfaces. In addition, to the implementation of improved cleaning regimes for the elimination of *L. monocytogenes*, finished product samples should be routinely monitored, since specifications for finished yeast product require the absence of *Listeria* in 25 g compressed and dry yeast used in baking and brewing (SABS 1642, 1995; SABS 1643, 1995).

The presence of spore formers found in dry yeast finished products in this study is undesirable, as the spores may survive in the vacuum-packaged dry yeast product, later endure the baking process and subsequently result in the rope-spoilage of commercially manufactured bread. If the bacterial spores are entering into the dry yeast product by means of surrounding processing air, the yeast factory should possibly reduce the flow of traffic around the dry yeast packaging line or shield the dry process and packaging line.

Figure 2.3.1 Rapid L' Mono agar (Bio-rad), a selective chromogenic medium, was used for the detection of *Listeria* (*L*.) *monocytogenes* from cream, compressed and dry yeast products. This picture shows a Rapid L' Mono agar plate with typical blue colonies of *L*. *monocytogenes*.

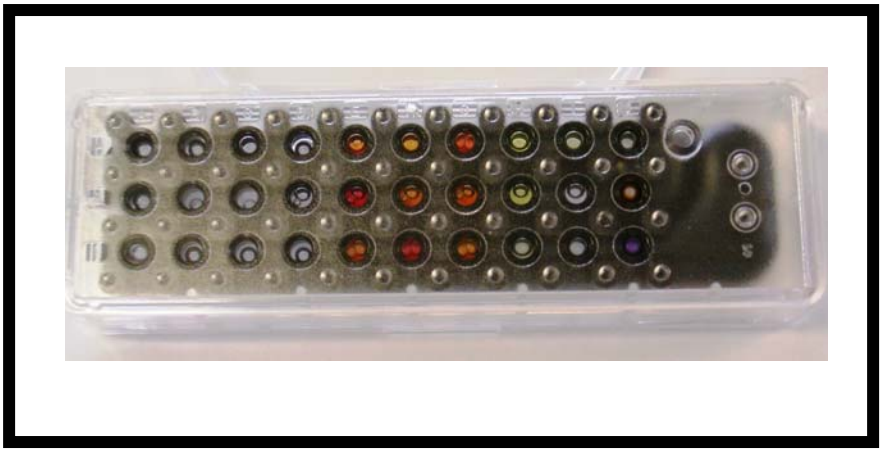


Figure 2.3.2 A BBL CRYSTAL™ Gram-Positive Identification system (Becton, Dickinson and Company) showing colour reactions with dehydrated substrates after inoculation and incubation at 37°C for 24 h.

Figure 2.3.3 Xylose – Lysine Desoxycholate (XLD) agar (Bio-rad) was used for the detection of *Salmonella* in cream, compressed and dry yeast products. This picture shows a XLD agar plate with typical red colonies with black centres of *Salmonella*.

Figure 2.3.4 3M™ Petrifilm™ Rapid *S. aureus* (RSA) count plates (3M) were used for the direct enumeration of *Staphylococcus* (*S.*) *aureus* from cream, compressed and dry yeast products. This picture shows a typical Petrifilm™ RSA count plate with red/ blue colonies of *S. aureus* surrounded by characteristic pink zones.

Figure 2.3.5 Bacterial spore counts associated with cream, compressed and dry yeast products were determined using Tryptone Soya agar (Oxoid) reinforced with 1.5% (w/ v) Bacteriological agar (Biolab). Aliquots of a 10^{0} (cream yeast) and 10^{-1} (compressed and dry yeast) dilution were heated at 80°C for 13 min prior to pour plating.

Table 2.3.1 Incidence[a] of *Listeria monocytogenes*, *Salmonella*, *Staphylococcus aureus* and bacterial spores in finished cream, compressed and dry yeast products, before and after preliminary incubation

Bacterial group	Cream Yeast		Compressed Yeast		Dry Yeast	
	Non-PI[b]	PI[c]	Non-PI	PI	Non-PI	PI
Presumptive *Listeria monocytogenes*	NF [d]	NF	1/3	2/3	2/3	3/3
Confirmed *Listeria monocytogenes*[e]	NF	NF	1/3	2/3	2/3	3/3
Bacterial spores	NF	NF	NF	NF	1/3	2/3
Confirmed *Bacillus* spp.[f]	NF	NF	NF	NF	1/3	2/3
Presumptive *Salmonella* spp.	NF	NF	NF	NF	NF	NF
Presumptive *S. aureus*	NF	NF	NF	NF	NF	NF

[a] Number of positives in 3 samples

[b] Samples analysed immediately after collection

[c] Samples analysed after preliminary incubation at 37°C for 24 h

[d] Not found

[e] Isolates that were Gram-positive, catalase-positive rods were confirmed as *L. monocytogenes*, using BBL CRYSTAL™ Gram-Positive Identification systems

[f] Isolates that were Gram-positive, catalase-positive rods were confirmed as *Bacillus* spp. using BBL CRYSTAL™ Gram-Positive Identification systems (Becton, Dickinson and Company)

CHAPTER THREE

MICROBIOLOGICAL SHELF-LIFE STUDIES ON COMMERCIALLY MANUFACTURED YEAST

ABSTRACT

This study was undertaken to determine the impact of storage temperature on the shelf-life of commercially manufactured yeast. During three replicate studies, cream, compressed and dry yeast samples were stored for 21 days at 4, 10, 25 and 37°C. At three day intervals, numbers of bacteria were quantified using standard plate counting procedures on various selective media, and 1044 predominant colonies isolated and characterized. The highest numbers of aerobic bacteria and *Enterococcus*, up to 7 and 8 log CFU/ ml or g, were found when cream and compressed yeast samples were exposed to elevated temperatures (25 and 37°C), while lower counts (4 and 6 log CFU/ ml or g) were obtained from samples kept refrigerated at 4°C. At 10°C, counts of aerobic bacteria and *Enterococcus* increased from 4 to 7 log CFU/ ml or g, highlighting the importance of temperature control during the storage and distribution of perishable cream and compressed yeast. At all temperatures, vacuum-packaging of dry yeast reduced *E. coli* and coliform counts and limited the growth of aerobic and *Enterococcus* counts to 4 log CFU/ g. Throughout storage, *Lactobacillus* dominated the bacterial populations in both cream and compressed yeast (45 to 78% of isolates), whilst *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*) predominated in vacuum-packaged dry yeast (54 to 68% of isolates). For all three yeast products, proportions of Gram-negative bacteria, *Micrococcus* and *Bacillus* recovered were low, suggesting they are not primarily responsible for the spoilage of commercial yeast products.

INTRODUCTION

Shelf-life can be defined as the period of time a product remains chemically, physically and microbiologically safe for human consumption and fit for use as a raw material when stored at the recommended storage temperature. Commercially propagated yeast is a living end-product and is highly nutritious, which inherently makes it susceptible to microbial spoilage, resulting in reduced shelf-life if not stored and distributed under appropriate conditions.

The microbial spoilage ecology of commercially manufactured yeast subsequent to initial contamination is influenced by a number of factors. Intrinsic, extrinsic or processing factors select which microbial populations which will predominate in the spoilage population. Intrinsic factors are the inherent qualities of a food product which influence the role and patterns of growth and are prime determinants in establishing the environment in which microorganisms compete for dominance. They include chemical, physical and biochemical characteristics such as nutrient composition, pH, water activity (a_w), and redox potential (Eh) of a food product (Jay, 1998). In order to grow and function normally, microorganisms require a source of water, energy, growth factors, vitamins and minerals (Jay, 1998). Nutrient-rich commercially manufactured yeast comprising of water (70%), proteins (15%), carbohydrates (10.5%), minerals (3%) and fats (1.5%), is highly susceptible to the contamination and growth of a variety of spoilage microorganisms (Reed and Nagodawithana, 1991).

It is well established that most microorganisms grow best at pH values around 7, and few grow below 4 (Jay, 1998). Bacteria, especially pathogenic species, tend to be more fastidious in their relationship to pH than moulds and yeasts (Jay, 1998). For less tolerant bacteria, adverse pH affects at least two aspects of a respiring microbial cell: the functioning of its enzymes and the transport of nutrients into the cell (Jay, 1998). The shelf-life of commercially manufactured yeast is often taken for granted due to the intrinsic preservative action which is incorporated during manufacturing by lowering the pH (*ca.* 5) (Reed and Nagodawithana, 1991). The intracellular pH of Baker's yeast cells is approximately 5.8, whereas the outer region of the cells during glucose fermentation is more acidic (Conway and Downy, 1950), making fresh cream and compressed

yeast susceptible to bacterial growth and spoilage by acid-tolerant members of the lactic acid bacteria (LAB) family (Chapter 2.2).

One of the oldest methods of preserving foods is drying or desiccation by the removal of moisture, without which microorganisms do not grow (Jay, 1998). In general, bacteria require higher values of a_w for growth than fungi, with Gram-negative bacteria having higher requirements than Gram-positives (Jay, 1998). Dry yeast production preserves yeast by desiccation, keeping the yeast for longer periods without any reduction in its fermenting activity (Walter, 1953). Redox potential is regarded as an intrinsic factor, but is influenced by extrinsic parameters such as packaging. Vacuum-packaging delays microbial spoilage by restricting the growth of spoilage organisms (Gill and Molin, 1991). The vacuum-packaging of dry yeast after flushing with nitrogen reduces the oxygen concentration in packs which retards the growth of aerobic or facultative anaerobic species, whilst enhancing the growth of microaerophiles. Some aerobic bacteria, for example *Lactobacillus* and *Campylobacter* spp., referred to as microaerophiles, grow better under slightly reduced oxygen conditions (Jay, 1998).

The extrinsic parameters of foods are those properties of the storage environment that affect both the foods and their microorganisms, and subsequent spoilage patterns. These include temperature of storage, relative humidity of the environment, presence and concentration of gases, and the presence and activities of other microorganisms (Jay, 1998). Microorganisms are capable of growth over a wide range of temperatures. The predominant spoilage population must be considered when selecting the proper temperature for the storage of a food product. The most predominant spoilage-causing bacteria are psychrotrophic, capable of growth at or below 7°C with optima between 20 and 30°C, such as *Lactobacillus*, *Micrococcus*, *Pseudomonas* and *Enterococcus* (Jay, 1998). These organisms grow well at low temperatures and are largely responsible for the spoilage of many refrigerated products including meats (Varnam, 2002), fish (Leroi *et al*., 1998), and poultry (Georgnaras *et al*., 1998). Since commercial cream and compressed yeast products are perishable, refrigeration between 2 and 4°C is required to retard the growth of most psychrotrophic species.

It is important that perishable food products are stored under conditions of relative humidity that do not allow the food to absorb moisture from the air and thereby increase its own surface and subsurface a_w to a point where microbial growth can occur (Jay, 1998). Foods that undergo surface spoilage from moulds, yeasts, and certain bacteria should be stored under conditions of low relative humidity. This is true for compressed yeast blocks, where moulds (e.g. *Penicillium* and *Fusarium*) will grow readily on the surface of the yeast cake if not stored at low temperatures and low humidity (Irvin, 1954; van Dam, 1986).

To meet customer demands, commercial yeast manufacturers must consistently produce a high-quality product with guaranteed storage-life. To date, limited work has been carried out on the bacteriological shelf-life of commercially manufactured yeast products. The objective of the current study was to determine the effects of various storage temperatures on the shelf-life and bacterial growth associated with commercially manufactured cream, compressed and dry yeast. In addition, this study aimed to establish whether bacterial counts associated with 'acceptable' and 'spoiled' yeast products corresponded with the recommended guidelines for the use of commercial yeast in baking and brewing (SABS 1642, 1995; SABS 1643, 1995). Accelerated shelf-life studies were carried out at elevated temperatures: 10°C (abuse temperature during transportation), 25 (wine making temperature) and 37°C (temperatures often encountered in bakeries), to simulate the spoilage of commercially manufactured yeast products in comparison to the effects of storage at the recommended temperature of 4°C.

In addition, this study examined the relationship between bacterial growth in commercially manufactured cream, compressed and dry yeast products and their sensory shelf-life. For the purposes of this work, sensory analysis was not a scientific assessment but rather a qualitative evaluation. Although there has been research on the bacterial populations associated with commercially manufactured compressed yeast blocks (Viljoen and Lues, 1993), limited work has been carried out on the bacterial populations associated with commercially manufactured cream and dry yeast products. In fact, the bacterial populations responsible for the spoilage of these three different commercially manufactured yeast products are largely unknown. This work also investigated the bacterial populations associated with the storage of commercially manufactured cream, compressed and dry yeast.

MATERIALS AND METHODS

(a) Sample description and collection

Three replicate shelf-life surveys were performed on finished cream, compressed and vacuum-packaged dry yeast products. For each study, 32 samples of each product were aseptically collected from a commercial yeast factory (Figure 3.1). Eight of each finished product type were incubated at 4, 10, 25 and 37°C. Samples were tested at 3 day intervals over a period of 21 days.

(b) Sample preparation for shelf-life analysis

On each sampling day, 10 ml of the cream yeast sample and 10 g of compressed or dry yeast sample were aseptically removed from one of the eight replicate samples stored at the different temperatures and prepared for bacteriological analysis (Chapter 2.1). Following preparation, homogenates of samples were serially diluted ten-fold in sterile buffered peptone water (Oxoid, Basingstoke, UK) and plated in duplicate using the standard pour-plate technique to enumerate *E. coli*, coliforms and *Enterococcus* and the spread-plate technique to enumerate aerobic plate count bacteria (Chapter 2.1, Table 2.1.2). In addition, the pH values of yeast samples were measured at each sampling interval after samples were removed for bacteriological analyses. The pH was measured at 25°C with a Metrohm 744 pH meter (Metrohm Ltd., Herisau, Switzerland). The pH was determined from the 10^0 dilution for cream yeast samples, and from the 10^{-1} dilution for both compressed and dry yeast samples. Sensory properties (appearance and odour acceptability) for cream, compressed and dry yeast samples were also recorded at each sampling interval.

(c) Bacterial enumeration and statistical analysis

For all analyses, plates containing between 30 and 300 colonies (or the highest number if below 30) were counted and the log colony forming units (CFU) per ml or gram determined. For each bacterial count type, standard deviations between the three replicate shelf-life studies were calculated. In addition, significant variances (ANOVA) between bacterial counts were

determined for all bacterial counts at the 95% confidence level using the STATGRAPHICS (STSC Inc. and Statistical Graphics Corporation) programme.

(d) Characterization of predominant spoilage populations

Bacterial populations were determined for each sample type and at each storage temperature for each day of sampling. Two predominant colonies were isolated from duplicate WL Nutrient agar (Oxoid) + 1% cyclohexamide (0.004g/ litre) (Scharlau, Barcelona, Spain) total aerobic plate count plates, using plates of highest dilutions showing growth (von Holy and Holzapfel, 1988). In this way, a total of 1044 isolates were obtained for cream (numbering 348), compressed (348) and dry (348) yeast samples stored at 4, 10, 25 and 37°C for 21 days. The bacterial isolates were purified and characterized to genus level using the dichotomous key of Fisher *et al.* (1986) described in Chapter 2.2.

RESULTS

Figures 3.2, 3.3 and 3.4 show the growth patterns of the different groups of bacteria during the storage of cream, compressed and dry yeast at 4, 10, 25 and 37°C for 21 days. Mean counts and standard deviations, as well as changes in pH for cream, compressed and dry yeast samples are shown. The recommended guidelines for *E. coli* (broken red line), coliforms (broken green line), *Enterococcus* (broken blue line) and aerobic bacteria (broken pink line) are shown in Figure 3.2, 3.3, 3.4 and Table 3.1 (SABS 1642, 1995; SABS 1643, 1995). The percentage incidence of *E. coli* and coliforms in cream, compressed and dry yeast products during storage are shown in Table 3.2 and Table 3.3.

(a) Bacterial counts associated with cream yeast storage

Immediately after production (day 0, 4°C) *Enterococcus* and aerobic counts for cream yeast were *ca*. 4 and 5 log CFU/ ml, respectively. At 4°C, *Enterococcus* counts reached their maximum level of 5 log CFU/ ml after 3 weeks, whilst aerobic counts gradually increased for the first 2 weeks of storage, reaching a maximum level of 7 log CFU/ ml (Figure 3.2, 4°C). Although, low storage

temperatures largely inhibited the growth of *E. coli* in cream yeast after 3 days (*ca.* 1 log CFU/ ml), significant growth ($P < 0.05$) was found for total coliforms after a prolonged lag phase (21 days) reaching 2 log CFU/ ml (Figure 3.2, 4°C). The storage of cream yeast at 10°C, resulted in increased *Enterococcus* and aerobic counts, reaching a maximum after 9 days of 7 and 8.5 log CFU/ ml, respectively (Figure 3.2, 10°C). *E. coli* growth was largely inhibited in cream yeast at 10°C (as observed at 4°C), however, coliform counts decreased from *ca*. 1.5 log CFU/ ml to below the lower detection limit (< 0.70 log CFU/ ml) after 15 days of storage. Exponential growth curves were observed for *Enterococcus* and aerobic bacteria after 6 days storage of cream yeast at 25 and 37°C reaching a maximum level of 7 to 7.5 and 8 to 9 log CFU/ ml, respectively. In addition, *E. coli* and coliforms became non-detectable (< 0.70 log CFU/ ml) in cream yeast product samples after 3 days at 37°C and 9 days at 25°C (Figure 3.2, 25 and 37°C).

The pH of cream yeast was fairly stable during storage at 4 and 10°C, ranging from 5 to 5.5 at both temperatures. Changes in pH of cream yeast stored at 25 and 37°C were more noticeable, ranging from 4.5 to 5.5 and 5 to 6, respectively (Figure 3.2). The results of sensory analyses demonstrated storage of cream yeast for 3 weeks at elevated temperatures resulted in the separation and settling of the yeast biomass, with pungent oudor and discolouration after 3 days at 37°C and 6 days at 25°C (Figure 3.5, A).

(b) Bacterial counts associated with compressed yeast storage

Similar to cream yeast counts, initial (day 0, 4°C) *Enterococcus* and aerobic counts for compressed yeast were *ca*. 4 and 4.5 log CFU/ g, respectively. *Enterococcus* counts remained stable (*ca*. 4 log CFU/ g) in compressed yeast samples stored at 4°C, while aerobic counts gradually increased reaching a maximum of *ca.* 6.5 log CFU/ g after 1 week (Figure 3.3, 4°C). After 3 days storage at 4°C and 10°C, coliform counts decreased from 2 log CFU/ g to 1 log CFU/ g. However, *E. coli* counts remained stable (*ca.* 1 log CFU/ g) at 4°C and 10°C after 21 days (Figure 3.3, 4°C and 10°C). Growth patterns for *Enterococcus* were very similar for compressed yeast samples stored at 10 and 25°C, reaching a maximum of 7 log CFU/ g after 15 days and 7.5 log CFU/ g after 6 days, respectively (Figure 3.3, 10°C and 25°C). At 10 and 25°C, aerobic counts had a high correlation with *Enterococcus* counts reaching a maximum of 8 and 8.5

log CFU/ g, respectively. Storage of compressed yeast at 37°C, resulted in shortened lag phase for *Enterococcus* and aerobic growth curves reaching a maximum of 7 log CFU/ g after 3 days and 8 log CFU/ g after 12 days, respectively (Figure 3.3, 37°C). However *Enterococcus* and aerobic counts significantly ($P < 0.05$) decreased during storage at 37°C after 12 days to 4 and 7 log CFU/ g, respectively. Limited growth for total coliforms and *E. coli* was evident after 3 to 6 days storage of compressed yeast samples at 25 and 37°C (Figure 3.3, 25°C and 37°C).

The pH of compressed yeast was fairly stable during storage at 4 and 10°C, ranging from 4 to 4.5 at both temperatures. Changes in pH of compressed yeast stored at 25 and 37°C were more noticeable (as observed in cream yeast), ranging from 4.5 to 5 and 4.5 to 6, respectively (Figure 3.3). The results of sensory analyses demonstrated at 4°C, the compressed yeast remained creamy white in colour and crumbly, with a fresh yeast smell. Compressed yeast samples stored at 10°C became "putty- like" after 9 days. At 25°C, compressed yeast samples became putty-like after 3 days and slimy with a pungent oudor after 9 days storage. At 37°C, compressed yeast samples changed colour to brown and liquefied after 6 days of storage with a pungent odour and slimy film (Figure 3.5, B).

(c) Bacterial counts associated with dry yeast storage

No significant growth ($P > 0.05$) of *Enterococcus* or aerobic bacteria was observed in vacuum-packaged dry yeast samples stored at 4, 10, 25 or 37°C. After 21 days of storage, *Enterococcus* and aerobic counts remained *ca.*4 and 4.5 log CFU/ g, respectively (Figure 3.4). Results showed the survival of *E. coli* and total coliforms at all storage temperatures *ca*. 1 and 2 log CFU/ g over 21 days, respectively.

The pH of dry yeast was stable during storage at all temperatures, ranging from 5.55 ± 0.20 to 5.96 ± 0.05 (Figure 3.4). There was no change in the appearance or oudor of dry yeast samples at 4, 10, 25 and 37°C after 21 days of storage. Dry yeast granules did not show any signs of spoilage, remaining cream coloured with a fresh yeast smell (Figure 3.5, C).

(d) Bacterial populations associated with commercial yeast products

The percentage distribution of predominant bacterial populations isolated from aerobic plate count plates of cream (Table 3.4), compressed (Table 3.5) and dry (Table 3.6) yeast samples during shelf-life studies are shown. Figure 3.6 shows the identity of 1044 bacteria isolated during shelf-life studies of cream, compressed and dry yeast.

Gram-positive bacteria predominated in samples of cream yeast, regardless of the storage temperature and number of days stored for (90.6 to 100%; Table 3.4). Cells of the Gram-positive bacterial genus predominantly recovered from cream yeast samples were rods of *Lactobacillus*, with proportions ranging from 61.5 to 77.4% (Figure 3.6, Table 3.4). The proportion of *Lactobacillus* isolated from cream yeast samples, increased with increased storage temperature (Figure 3.6, Table 3.4). In general, *Lactobacillus* predominated over *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*) (15.5 to 27.3%), Gram-negative rods (3.6 to 9.4%), *Micrococcus* (1.2 to 5.2%) and *Bacillus* (1 to 1.2%), respectively (Figure 3.6, Table 3.4). In cream yeast, the highest proportions of *Lactobacillus* were recovered at 25°C, *Enterococcaceae* at 10°C, *Micrococcus* at 4°C, *Bacillus* at 10 and 25°C, and Gram-negative rods at 4°C (Figure 3.6, Table 3.4). The loss of sensory properties in cream yeast samples when stored at 25 and 37°C correlated with high proportions of *Lactobacillus* (*ca*. 70%).

As observed in cream yeast shelf-life studies, Gram-positive bacteria also predominated in samples of compressed yeast, regardless of the storage temperature and time (90.5 to 98.8%; Table 3.5). Gram-positive rods predominated over cocci with increasing storage temperature comprising 46.9 to 71.4% of the total population (Figure 3.6, Table 3.5). Similar to cream yeast, *Lactobacillus* was the predominant Gram-positive rod recovered from compressed yeast samples and proportions increased with increased storage temperature from 4 to 37°C (44.8 to 70.2%, Figure 3.6 and Table 3.5). In general *Lactobacillus* predominated over *Enterococcaceae* (16.7 to 42.9%), *Micrococcus* (2.4 to 9.4%), Gram-negative rods (1.2 to 9.5%), and *Bacillus* (1.2 to 2.1%), respectively (Table 3.5). In compressed yeast, the highest proportions of *Lactobacillus* were recovered at 37°C, *Enterococcaceae* at 10°C, *Micrococcus* at 4°C, Gram-negative rods at 37°C and *Bacillus* at 4°C (Figure 3.6, Table 3.5). Similar to cream yeast, the loss of sensory

properties and visual spoilage of compressed yeast samples when stored at 25 and 37°C correlated with high proportions of *Lactobacillus* (*ca*. 65%).

Gram-positive bacteria predominated in all dry yeast samples (as observed in cream and compressed yeast) (90.5 to 98.8%; Table 3.6). However, for dry yeast samples populations were dominated by Gram-positive cocci (59.4 to 71.4%; Table 3.6). In general, the proportion of Gram-positive cocci increased with an increase in storage temperature (from 4 to 37°C), whilst the proportion of Gram-positive rods decreased. In general, *Enterococcaceae* (54.2 to 67.8%) predominated over *Lactobacillus* (22.6 to 35.7%), *Micrococcus* (3.6 to 6%), Gram-negative rods (1.2 to 7.1%), and *Bacillus* (1.2 to 2.4%), respectively (Figure 3.6, Table 3.6). In dry yeast, the highest proportions of *Enterococcaceae* were recovered at 25°C, *Lactobacillus* at 10°C, *Micrococcus* at 10°C, Gram-negative rods at 37°C, and *Bacillus* at 37°C (Figure 3.6, Table 3.6). In contrast with the predominance of *Lactobacillus* in cream and compressed yeast samples, higher proportions of *Enterococcaceae* were consistently isolated from dry yeast samples (Figure 3.6).

DISCUSSION

Bacterial counts associated with the storage of commercially manufactured yeast

The purpose of this work was to investigate quantitative and qualitative evolution in the bacterial populations associated with commercially manufactured cream, compressed and dry yeast products during storage at various temperatures. For all sample types, aerobic counts had a high correlation with *Enterococcus* counts, which were consistently 3-to 4-fold higher than coliform and *E. coli* counts, respectively.

The shape of a bacterial growth curve is affected by factors such as temperature and limitation of nutrients (Shapton and Shapton, 1991). For cream and compressed yeast samples, growth of *Enterococcus* and aerobic bacteria was observed at all storage temperatures, exhibiting typical growth curves, with the slope of the exponential growth phase greater at 25 and 37°C (3 to 6 days) than at 4 and 10°C (9 to 15 days). Increases in counts at 4°C were attributed to the growth

of psychrotrophic bacteria, capable of multiplying at low temperatures (Hartman *et al.*, 1992). Reportedly, LAB, including *Enterococcus* spp., are relatively resistant to chilling and can grow at temperatures as low as 10°C (van Dam, 1986; Collee *et al*., 1996), and can thus be expected to survive the cold-storage of commercial cream and compressed yeast products. Psychrotrophic bacteria metabolize carbohydrates, proteins, and lipids across a range of temperatures, but reaction rates are generally slower at temperatures below 7°C (Hartman *et al.*, 1992). In this study *Enterococcus* and aerobic counts were 1 to 2 log higher in samples stored at 10°C than at 4°C, demonstrating the effect a break in the cold-chain during storage and distribution (by *ca*. 5°C) can have on the shelf-life of fresh yeast products. Gassing measurements found that the activity of cream and compressed yeast did not significantly decrease ($P > 0.05$) upon extended storage at 4°C (data not shown). This was in agreement with previous studies of Baker's compressed yeast stored at various temperatures, which found no loss in activity after four weeks at 5°C (Hautera and Lovgren, 1975). At elevated storage temperatures such as 25 and 37°C, the rapid exponential growth of *Enterococcus* and aerobic bacteria in cream and compressed yeast was expected (Shapton and Shapton, 1991). The visual spoilage of perishable cream and compressed yeast product samples stored at 25 and 37°C correlated with high levels of aerobic bacteria and *Enterococcus* which stabilized after 3 to 6 days (*ca.* 8 log CFU/ ml or g).

Results obtained in this study found coliform and *E. coli* counts exceeded the recommended guidelines in 13 and 100% of refrigerated samples, respectively. This suggested low levels of *E. coli* and coliforms were capable of survival or slow growth during cold-storage of cream and compressed yeast at 4 and 10°C. Reportedly, coliforms, such as *E. coli*, can grow at temperatures as low as 10°C (Prescott *et al*., 1996). In contrast, at higher storage temperatures, coliforms and *E. coli* became non-detectable (< 0.70 log CFU/ ml or g) in cream and compressed yeast samples when stored for more than 6 days at 25°C and 3 days at 37°C. This phenomenon could be due to (i) competition with members of the LAB family, which not only outgrow other bacterial contaminants in numbers at elevated temperatures but also (ii) decrease the pH of their surroundings through the metabolic production of lactic acid making conditions less favourable for the growth of other bacteria and (iii) produce bacteriocins, such as nisin, which inhibit the growth of predominantly Gram-negative bacteria (Jay, 1998).

Sensory analysis confirmed that numbers of aerobic bacteria and *Enterococcus* in cream and compressed yeast could be very high before product was visually spoiled. Cream yeast biomass settled after 1 to 3 days storage at all four temperatures, due to the lack of agitation which would otherwise maintain the yeast cells in suspension. The spoilage of cream yeast at elevated temperatures resulted in discolouration, pungent and rancid odours. Guidelines for the Baker's compressed yeast requires that the yeast be light cream in colour, with a characteristic yeasty odour and taste, showing no signs of deterioration (SABS 1642, 1995). At elevated storage temperatures, the combined sensory results from three studies found a progressive visual pattern for the spoilage of compressed yeast which was initiated by the softening of the yeast cake with a 'putty-like' texture. This was followed by slime formation and stickiness. Reportedly, LAB are responsible for the formation of slime which can adversely affect the gassing activity of yeast and often results in economic losses due to premature spoilage (Tessendorf, 1991). The yeast later became discoloured with a pungent, rancid odour and in time liquefied due to the autolysis of yeast cells. Fresh yeast products are sensitive to temperature changes, and when exposed to unfavourable climatic conditions (normally greater than 15°C) for any length of time, autolytic endogenous enzymes produced by the yeast cells degrade the cell plasma and the yeast is decomposed by a process known as autolysis (Walter, 1953; Reed and Nagodawithana, 1991). Similar to the spoilage pattern of compressed yeast found in this study, previous studies of vacuum-packaged vienna sausages have reported that LAB are responsible for spoilage causing undesirable souring, 'off' odours, slimy and milky extrudates (Dykes *et al*., 1996).

Previous shelf-life studies of packaged products, for example milk, found that the opening of packaging increases the availability of oxygen resulting in higher bacterial counts (Duyvesteyn *et al*., 2001). In this study, separate unopened vacuum-packaged dry yeast samples were tested at each sampling interval, in order to eliminate any growth resulting from an increase in oxygen availability. For dry yeast samples, all bacterial counts were not significantly different ($P > 0.05$) between storage temperatures over the 21 day period. This confirmed that drying and vacuum-packaging of yeast limits the growth and proliferation of bacterial contaminants (Reed and Nagodawithana, 1991). In a previous study of the survival of bacteria in spray-dried powders used for the production of microbiological reference materials, Gram-positive strains such as *Enterococcus* showed relatively high resistance to desiccation with more than 30% survival on

each day of sampling, whilst Gram-negative bacteria, such as *E. coli* showed a lower resistance (Janning and Veld, 2001). Of the 32 dry yeast samples analysed at different storage temperatures, 100% contained coliforms, demonstrating their ability to survive under dry conditions and vacuum-packaging (Hartman *et al*., 1992). After 3 days of storage, *E. coli* counts decreased significantly ($P < 0.05$) from *ca.* 2 to 1 log CFU/ g, suggesting a marked reduction in *E. coli* after vacuum-packaging of dry yeast.

In addition to being bacteriologically stable, dry yeast when stored with a protective inert atmosphere and vacuum-packaging, only loses ± 1% of its fermentation activity per month, and less than 10% per year (Reed and Nagodawithana, 1991). In this study, dry yeast stored at 25°C for 21 days, had a fairly stable gassing activity of 107.67 ± 10.13 ml/ CO_2 at 120 minutes (data not shown). Guidelines for Brewer's dry yeast requires that the yeast be in the form of cream coloured powder or granules, have an oudor characteristic of good Brewer's yeast, and show no signs of deterioration (SABS 1643, 1995). All dry yeast samples in this study complied with these guidelines regardless of storage temperature or time. In fact, no signs of spoilage, change in appearance or odour were noted for any of the dry yeast samples after 21 days. Our results for the bacteriological shelf-life and functionality of commercial yeast products therefore show dry yeast is a better product than cream and compressed yeast in terms of its stability, fermentative activity and longevity.

Bacterial populations associated with commercially manufactured yeast

For cream, compressed and dry yeast products, bacterial populations isolated from aerobic plate counts of shelf-life samples at all four temperatures consisted predominantly Gram-positive genera (90.5 to 100%) of *Lactobacillus* and *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*), followed by *Micrococcus*, Gram-negative bacteria and *Bacillus*, respectively. At the recommended storage temperature, the percentage proportion of *Lactobacillus* decreased with decreased a_w of the yeast product, such that *Lactobacillus* comprised 61.5%, 44.8% and 26.2% of the total bacterial population associated with cream, compressed and dry yeast, respectively. The opposite was true for *Enterococcaceae*, whose proportion increased with decreased a_w of the yeast product, such that

Enterococcaceae comprised 22.9%, 39.6% and 67.8% of the total bacterial population associated with cream, compressed and dry yeast, respectively. These findings suggested that the survival and growth of *Lactobacillus* and *Enterococcaceae* may be dependent upon the a_w of commercially manufactured yeast.

In all three yeast products stored at various temperatures, LAB quickly became the dominant bacterial population, possibly due to their predominance in the bacterial population residing within the finished products (Chapter 2.2) (Reed and Nagodawithana, 1991; Viljoen and Lues, 1993; O'Brien *et al*., 2004a). Our results were in agreement with previous studies which found the genus *Lactobacillus* to predominate in Baker's compressed yeast comprising 84.1% of the total bacterial population (Viljoen and Lues, 1993). Our results suggest lactobacilli may be the main spoilage bacteria in fresh yeast as the proportion of lactobacilli increased during the storage of cream and compressed yeast at elevated temperatures (e.g. 25 and 37°C) which correlated with increased aerobic counts and the sensory deterioration of cream and compressed yeast products.

A noteworthy finding was the small proportion of Gram-negative isolates in predominant populations of visually spoiled cream and compressed yeast (*ca.* 4%). These results suggest Gram-negative bacteria may not be responsible for the spoilage of commercially manufactured cream and compressed yeast, but should rather be recognized for their role as indicators of failed sanitation. The overall reduction of Gram-negative rods throughout the storage of cream and compressed yeast, especially at elevated storage temperatures that favour the growth of LAB (e.g. 25 to 30°C), suggested that the high proportions of LAB may reduce the growth or survival of Gram-negative bacteria in commercially manufactured fresh cream and compressed yeast throughout storage. This is not the first time LAB have been recognized for their beneficial role in reducing the growth of unwanted microorganisms. Reportedly, LAB dominate most spoilage associations, often to the exclusion of other potential spoilage bacteria and foodborne pathogens (Dykes *et al*., 1993)

For both cream and compressed yeast samples, proportions of *Enterococcaceae* were higher in samples stored at 10°C, than at any other storage temperature. These results suggested that enterococci, belonging to the *Enterococcaceae* group, which characteristically grow at

temperatures as low as 10°C (Devriese *et al*. 1991, Manero and Blanch, 1999; Franz *et al.*, 2003) may constitute the predominant spoilage population of refrigerated cream and compressed yeast products. This statement is further supported by the high correlation between aerobic and *Enterococcus* counts obtained in this study. Similarly, low storage temperatures (4°C) favoured the growth of *Micrococcus* in cream and compressed yeast whose proportion within the bacterial population increased to 5.2 and 9.4% respectively. In general, micrococci have little pathogenic potential (Sleigh and Timbury, 1998), and their spoilage potential of refrigerated products is largely unknown. These results indicate the importance of maintaining the cold-chain throughout the production and storage of fresh cream and compressed yeast, since a rise in temperature to 10°C could promote the unfavourable growth of *Enterococcaceae*, such as enterococci, and *Micrococcus* spp.

The drying of filtered cake into dry yeast at ± 55°C is not sufficiently high enough to eliminate heat sensitive mesophiles, which survive the drying process and reside within the finished dry yeast product during subsequent storage under vacuum-packaged conditions. Shelf-life studies of vacuum-packaged dry yeast, found Gram-positive LAB predominated at all storage temperatures. It is well documented that vacuum-packaging can promote the development of LAB since they are facultative anaerobic bacteria (Dykes *et al*., 1993; Leroi *et al*., 1998). Furthermore, our results suggest Gram-positive cocci belonging to the LAB family, may be more resilient than Gram-positive rods to the preserving effect of drying and vacuum-packaging. Reportedly, the major bacterial genera found in vacuum or modified atmospheric packaged foods include Gram-positive cocci belonging to the genera, *Leuconostoc* and *Pediococcus* (Hartman *et al.*, 1992).

The recovery of Gram-negative bacteria from dry yeast samples was infrequent, being isolated from 34% of all samples. Gram-negative bacteria are predominantly aerobic organisms, suggesting vacuum-packaging may have inhibited their growth. Previous studies on the survival of Gram-negative bacteria after vacuum-packaging, found vacuum storage decreases the concentration of oxygen while the concentration of carbon dioxide (CO_2), inhibitory to Gram-negative bacteria, gradually selects for CO_2-tolerant Gram-positive bacteria (Gill and Tan, 1980; Leroi *et al*., 1998; Guynot *et al*., 2003). The preserving effect of vacuum-packaging or modified atmosphere packaging varies with concentration, incubation temperature, organism, and the a_w of

the product (Faber, 1991; Guynot *et al*., 2003). Thus, the effect of incubation temperature may explain the increased incidence of Gram-negative bacteria in vacuum-packaged dry yeast samples stored at 37°C in this study (63% of samples). *Bacillus* was isolated from 12.5% of dry yeast samples, comprising 1.2 to 2.1% of the total population. This was in agreement with spore counts obtained for finished dry yeast products (Chapter 2.3) and previous findings of the presence of rope-causing *Bacillus* in commercial yeast used for baking (Viljoen and von Holy, 1997).

CONCLUSION

Our study showed that various storage temperatures and times noticeably influenced the bacterial growth patterns associated with commercially manufactured fresh yeast. Cream and compressed yeast samples became bacteriologically and visually spoiled over time when stored at elevated storage temperatures (10, 25 and 37°C). Enterococci, characteristically capable of growth at 10°C, multiplied in refrigerated cream and compressed yeast samples, reinforcing the importance of maintaining the cold-chain throughout production, storage and distribution, which could otherwise result in premature product spoilage and economic loss to the manufacturer. Vacuum-packaged dry yeast remained bacteriologically stable throughout this study, whilst maintaining good sensory properties. In addition, the well documented effects of vacuum-packaging on the survival of foodborne spoilage and pathogenic bacteria was observed in this study, where counts of Gram-negative coliforms, specifically *E. coli*, were largely reduced in vacuum-packaged dry yeast. Population results from this study were not in agreement with previous literature (Reed and Nagodawithana, 1991) which found finished dry and fresh yeast products to contain the same predominant bacterial populations. Our findings suggested that populations of *Enterococcaceae*, including enterococci, predominated in finished dry yeast product, whilst *Lactobacillus* dominated the bacterial population associated with cream and compressed yeast products.

Figure 3.1 The appearance of three commercial yeast products prepared for shelf-life studies. **(A)** Individual 500 g bricks of vacuum-packaged dry yeast were used for shelf-life tests. **(B)** Finished cream yeast product was aseptically collected from the storage tank and divided into 30 ml volumes in sterile sampling bottles for shelf-life tests. **(C)** The 5kg finished compressed yeast brick was aseptically divided into 250 g portions and wrapped in wax paper obtained from the yeast manufacturing plant for shelf-life tests.

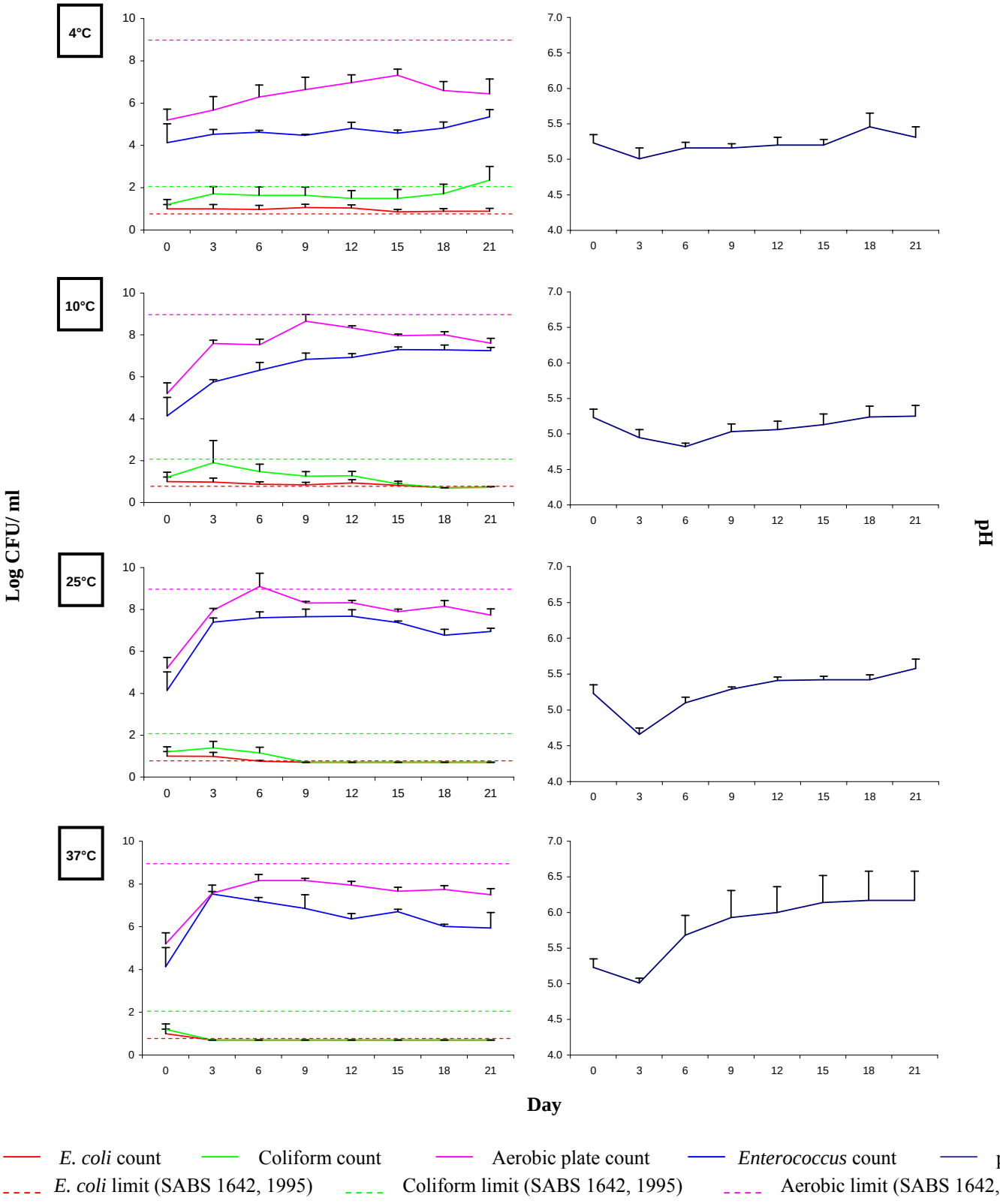


Figure 3.2 Mean *E. coli*, coliform, *Enterococcus*, aerobic plate counts (Log CFU/ ml) and pH of cream yeast product samples after 21 days storage at 4, 10, 25 and 37°C over three replicate surveys (Lower detection limit = 0.7 log CFU/ ml).

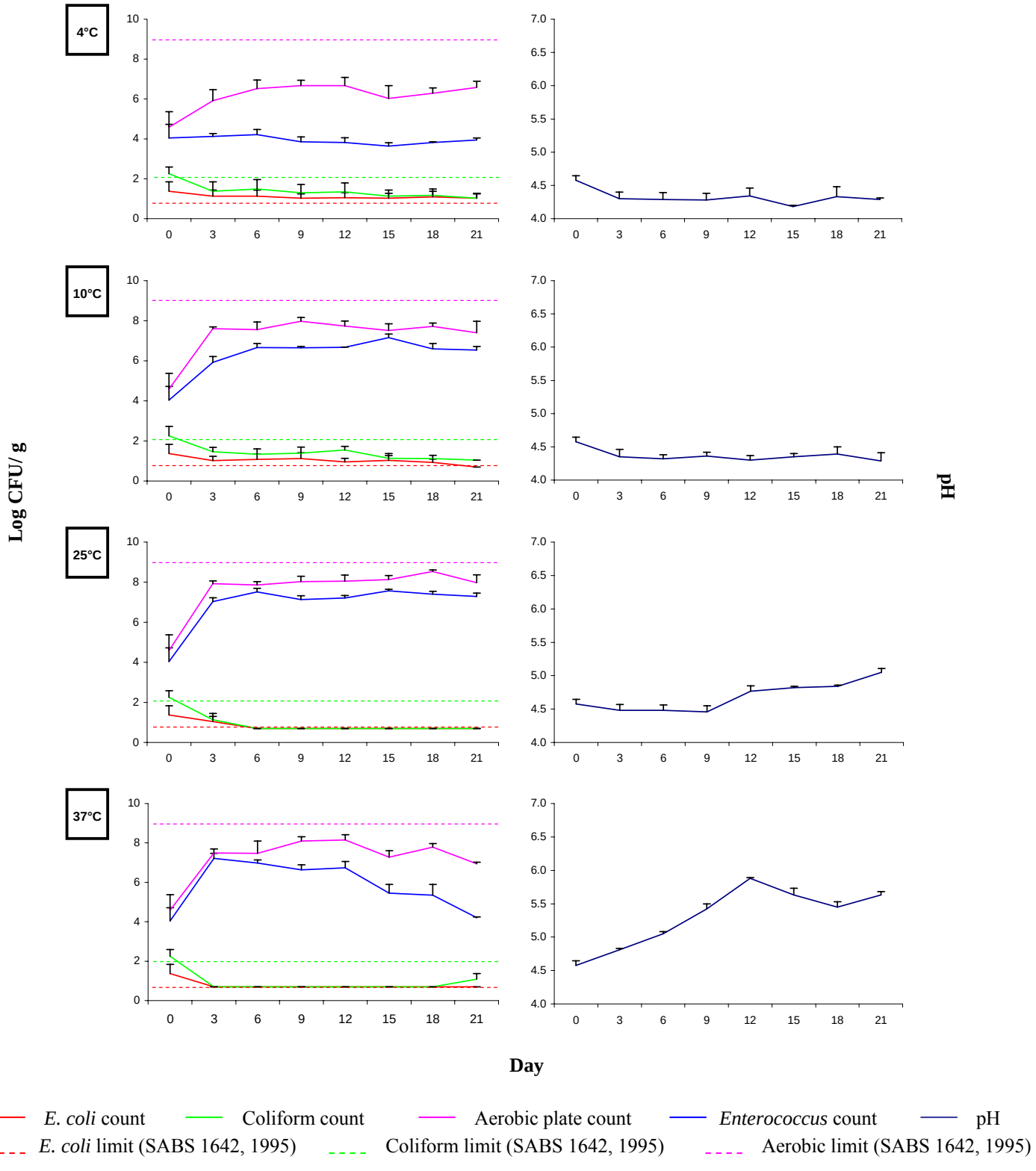


Figure 3.3 Mean *E. coli*, coliform, *Enterococcus*, aerobic plate counts (Log CFU/ ml or g) and pH of compressed yeast product samples after 21 days storage at 4, 10, 25 and 37°C over three replicate surveys (Lower detection limit = 0.7 log CFU/g).

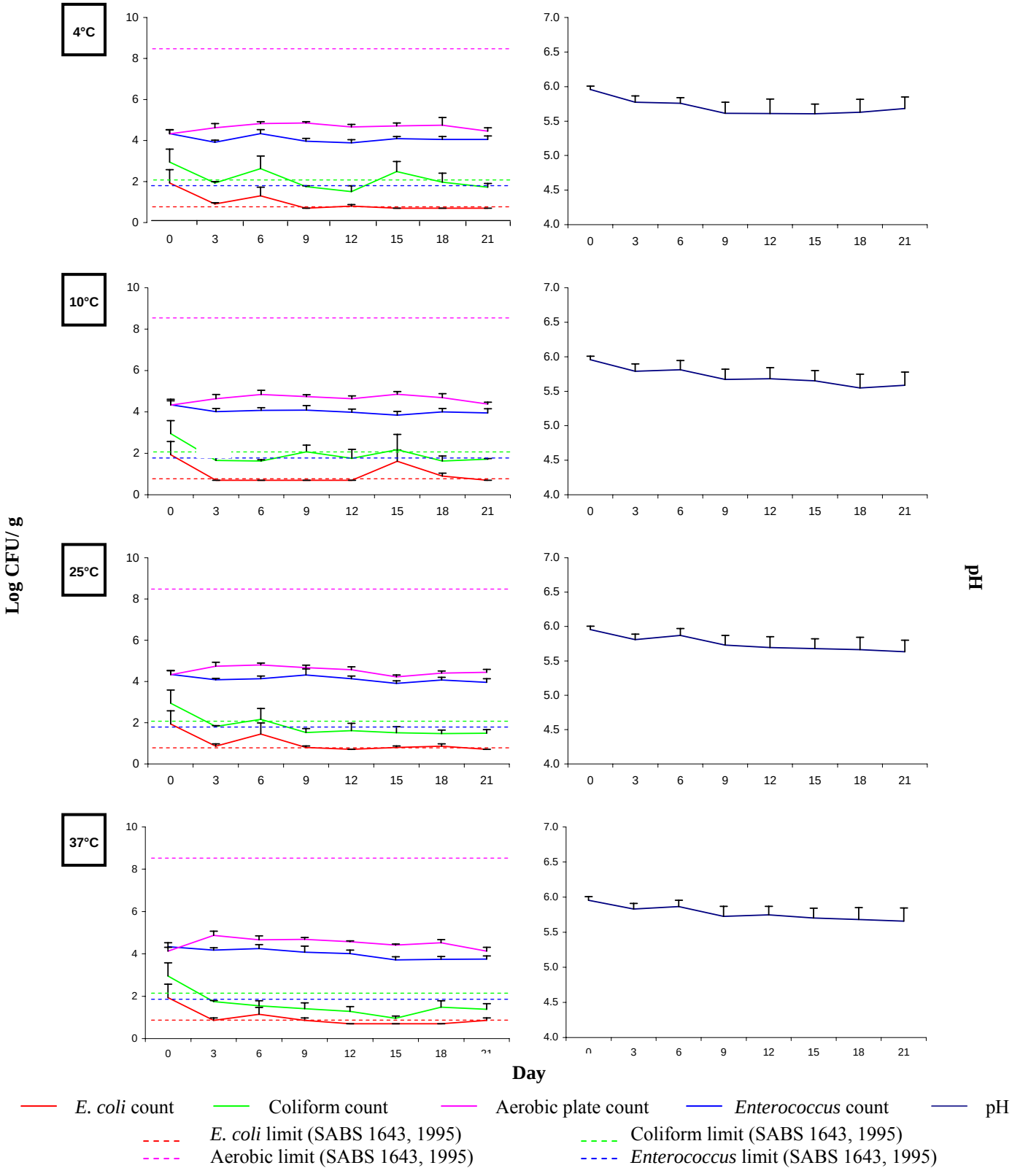


Figure 3.4 Mean *E. coli*, coliform, *Enterococcus*, aerobic plate counts (Log CFU/ ml or g) and pH of dry yeast product samples after 21 days storage at 4, 10, 25 and 37°C over three replicate surveys (Lower detection limit = 0.7 log CFU/ g).

Table 3.1 Recommended bacteriological guidelines for Baker's compressed yeast and Brewer's dry yeast (SABS 1642, 1995; SABS 1643, 1995)

Finished Product	Bacteria (log CFU/ g or ml)			
	E. coli	**Coliform**	***Enterococcus***	**Total bacteria**
Baker's Yeast	0	< 2	–	< 9
Brewer's Yeast	0	< 2	< 1.7	< 8.5

Table 3.2 Percentage incidence of *E. coli* in 32 cream, compressed and dry yeast samples stored at 4, 10, 25 and 37°C for 21 days

Product type	Storage Temperature (°C)				Total
	4	**10**	**25**	**37**	
Cream yeast	100[a]	88	25	13	56
	(100)[b]	(88)	(25)	(13)	(56)
Compressed yeast	100	75	38	13	56
	(100)	(75)	(38)	(13)	(56)
Dry yeast	38	38	75	63	53
	(38)	(38)	(75)	(63)	(53)
Cream, compressed and dry yeast	60	50	34	22	41
	(60)	(50)	(34)	(22)	(41)

[a] Percentage of samples in which *E. coli* was detected

[b] Percentage of samples in which *E. coli* counts exceeded the recommended limit (absent in 1 ml or g) (SABS 1642, 1995; SABS 1643, 1995)

Table 3.3 Percentage incidence of coliforms in 32 cream, compressed and dry yeast samples stored at 4, 10, 25 and 37°C for 21 days

Product type	Storage Temperature (°C)				Total
	4	**10**	**25**	**37**	
Cream yeast	100[a]	88	38	13	60
	(13)[b]	(0)	(0)	(0)	(3)
Compressed yeast	100	100	25	100	81
	(13)	(13)	(13)	(13)	(13)
Dry yeast	100	100	100	100	100
	(38)	(38)	(25)	(13)	(28)
Cream, compressed and dry yeast	100	72	41	53	66
	(16)	(13)	(19)	(6)	(14)

[a] Percentage of samples in which *E. coli* was detected

[b] Percentage of samples in which *E. coli* counts exceeded the recommended limit (absent in 1 ml or g) (SABS 1642, 1995; SABS 1643, 1995)

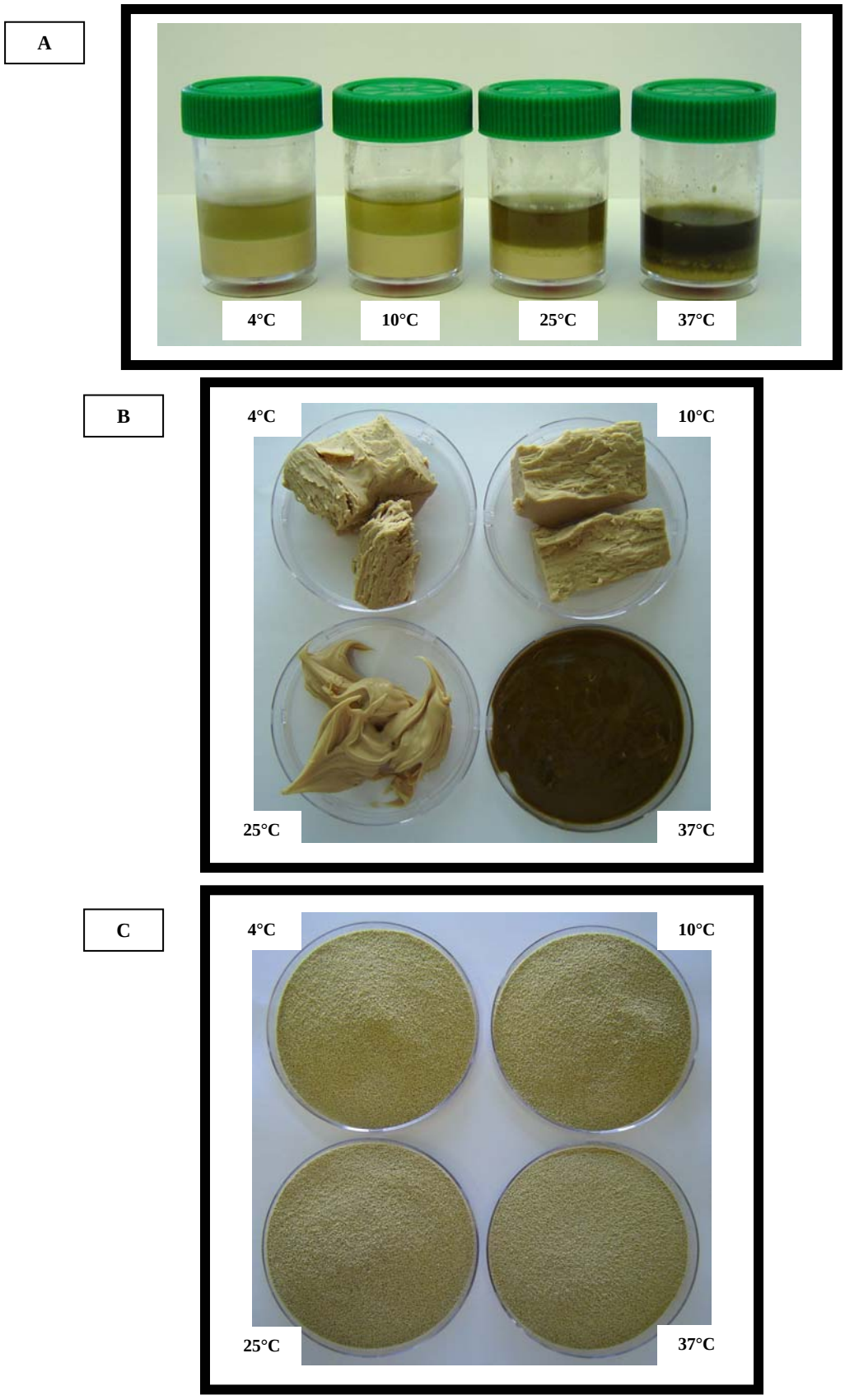


Figure 3.5 The appearance of cream **(A)**, compressed **(B)**, and dry yeast **(C)** after 21 days storage at 4, 10, 25 and 37°C. **(A)** The separation of cream yeast and product discolouration with increased storage temperature can be seen. **(B)** The visual pattern of spoilage of compressed yeast product ('putty-like' at 10°C, slimy at 25°C and liquefied and discoloured at 37°C). **(C)** No visual change in dry yeast samples during shelf-life studies.

Table 3.4 Percentage distribution of 348 isolates from aerobic plate counts of cream yeast samples stored at 4, 10, 25 and 37°C for 21 days

4°C

Day	% *Lactobacillus*	% *Enterococcaceae*	% *Micrococcus*	% *Bacillus*	% Gram negative
0	41.7	41.7	8.3	0	8.3
3	75	8.3	8.3	0	8.3
6	75	25	0	0	0
9	75	25	0	0	0
12	58.3	33.3	0	0	8.3
15	66.7	16.7	0	0	16.7
18	66.7	8.3	16.7	8.3	0
21	33.3	25	8.3	0	33.3
TOTAL = 96	**61.5**	**22.9**	**5.2**	**1.0**	**9.4**
% Gram-positive	**90.6 (62.5 % rods and 28.1 % cocci)**				

10°C

Day	% *Lactobacillus*	%*Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram negative
3	50	41.7	0	8.3	0
6	58.3	33.3	0	0	8.3
9	83.3	8.3	0	0	8.3
12	75	16.7	0	0	8.3
15	75	25	0	0	0
18	33.3	50	16.7	0	0
21	83.3	16.7	0	0	0
TOTAL = 84	**65.5**	**27.3**	**2.4**	**1.2**	**3.6**
% Gram-positive	**96.4 (66.7 % rods and 29.7 % cocci)**				

25°C

Day	% *Lactobacillus*	% *Enterococcaceae*	% *Micrococcus*	% *Bacillus*	% Gram negative
3	50	41.7	0	8.3	0
6	66.7	33.3	0	0	0
9	75	25	0	0	0
12	75	25	0	0	0
15	100	0	0	0	0
18	75	16.7	8.3	0	0
21	100	0	0	0	0
TOTAL = 84	**77.4**	**20.2**	**1.2**	**1.2**	**0**
% Gram-positive	**100 (78.6 % rods and 21.4 % cocci)**				

37°C

Day	% *Lactobacillus*	% *Enterococcaceae*	% *Micrococcus*	% *Bacillus*	% Gram negative
3	75	8.3	16.7	0	0
6	75	8.3	0	0	16.7
9	100	0	0	0	0
12	66.7	16.7	8.3	0	8.3
15	66.7	25	8.3	0	0
18	58.3	16.7	8.3	0	16.7
21	66.7	33.3	0	0	0
TOTAL = 84	**72.6**	**15.5**	**5.9**	**0**	**6.0**
% Gram-positive	**94 (72.6 % rods and 21.4 % cocci)**				

[a] *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)

Table 3.5 Percentage distribution of 348 isolates from aerobic plate counts of compressed yeast samples stored at 4, 10, 25 and 37°C for 21 days

	Day	% *Lactobacillus*	%*Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram negative
4°C	**0**	25	66.7	0	0	8.3
	3	50	33.3	16.7	0	0
	6	33.3	33.3	25	0	8.3
	9	33.3	50	8.3	0	8.3
	12	50	33.3	8.3	0	8.3
	15	50	25	16.7	8.3	0
	18	50	41.7	0	8.3	0
	21	66.7	33.3	0	0	0
	TOTAL = 96	**44.8**	**39.6**	**9.4**	**2.0**	**4.2**
	% Gram-positive	**95.8 (46.9 % rods and 49 % cocci)**				
10°C	**Day**	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
	3	16.7	66.7	8.3	0	8.3
	6	33.3	58.3	8.3	0	0
	9	50	50	0	0	0
	12	50	16.7	25	8.3	0
	15	58.3	33.3	8.3	0	0
	18	58.3	41.7	0	0	0
	21	66.7	33.3	0	0	0
	TOTAL = 84	**47.6**	**42.9**	**7.1**	**1.2**	**1.2**
	% Gram-positive	**98.8 (48.8 % rods and 50 % cocci)**				
25°C	**Day**	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
	3	41.7	50	8.3	0	0
	6	50	50	0	0	0
	9	66.7	16.7	0	8.3	8.3
	12	66.7	16.7	8.3	0	8.3
	15	66.7	16.7	8.3	0	8.3
	18	75	25	0	0	0
	21	75	16.7	8.3	0	0
	TOTAL = 84	**63.1**	**27.4**	**4.7**	**1.2**	**3.6**
	% Gram-positive	**96.4 (64.3 % rods and 32.1 % cocci)**				
37°C	**Day**	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
	3	66.7	16.7	0	0	16.7
	6	66.7	8.3	0	8.3	16.7
	9	66.7	25	8.3	0	0
	12	75	25	0	0	0
	15	83.3	8.3	0	0	8.3
	18	66.7	16.7	0	0	16.7
	21	66.7	16.7	8.3	0	8.3
	TOTAL = 84	**70.2**	**16.7**	**2.4**	**1.2**	**9.5**
	% Gram-positive	**90.5 (71.4 % rods and 19.1 % cocci)**				

[a] *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)

Table 3.6 Percentage distribution of 348 isolates from aerobic plate counts of dry yeast samples stored at 4, 10, 25 and 37°C for 21 days

	Day	% *Lactobacillus*	%*Enterococcaceae*[a]	% *Micrococcus*	% *Bacillus*	% Gram negative
4°C	**0**	33.3	58.4	0	8.3	0
	3	8.3	83.3	8.3	0	0
	6	41.7	58.3	0	0	0
	9	75	25	0	0	0
	12	16.7	58.3	16.7	0	8.3
	15	66.7	16.7	0	0	16.7
	18	8.3	75	8.3	8.3	0
	21	25	58.3	8.3	0	8.3
	TOTAL = 96	**34.3**	**54.2**	**5.2**	**2.1**	**4.2**
	% Gram-positive	**95.8 (36.4 % rods and 59.4 % cocci)**				
	Day	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
10°C	**3**	50	33.3	0	8.3	8.3
	6	41.7	50	8.3	0	0
	9	50	41.7	0	0	8.3
	12	41.7	50	8.3	0	0
	15	16.7	66.7	16.7	0	0
	18	16.7	75	8.3	0	0
	21	33.3	66.7	0	0	0
	TOTAL = 84	**35.7**	**54.8**	**5.9**	**1.2**	**2.4**
	% Gram-positive	**97.6 (36.9 % rods and 60.7 % cocci)**				
	Day	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
25°C	**3**	16.7	83.3	0	0	0
	6	33.3	66.7	0	0	0
	9	8.3	91.7	0	0	0
	12	33.3	66.7	0	0	0
	15	41.7	41.7	8.3	8.3	0
	18	25	75	0	0	0
	21	25	50	16.7	0	8.3
	TOTAL = 84	**26.2**	**67.8**	**3.6**	**1.2**	**1.2**
	% Gram-positive	**98.8 (27.4 % rods and 71.4 % cocci)**				
	Day	**% *Lactobacillus***	**% *Enterococcaceae***	**% *Micrococcus***	**% *Bacillus***	**% Gram negative**
37°C	**3**	0	91.7	8.3	0	0
	6	16.7	66.7	0	0	16.7
	9	33.3	58.3	0	0	8.3
	12	8.3	66.7	16.7	0	8.3
	15	16.7	58.3	8.3	16.7	0
	18	33.3	58.3	0	0	8.3
	21	50	33.3	8.3	0	8.3
	TOTAL = 84	**22.6**	**61.9**	**6.0**	**2.4**	**7.1**
	% Gram-positive	**92.9 (25 % rods and 67.9 % cocci)**				

[a] *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*)

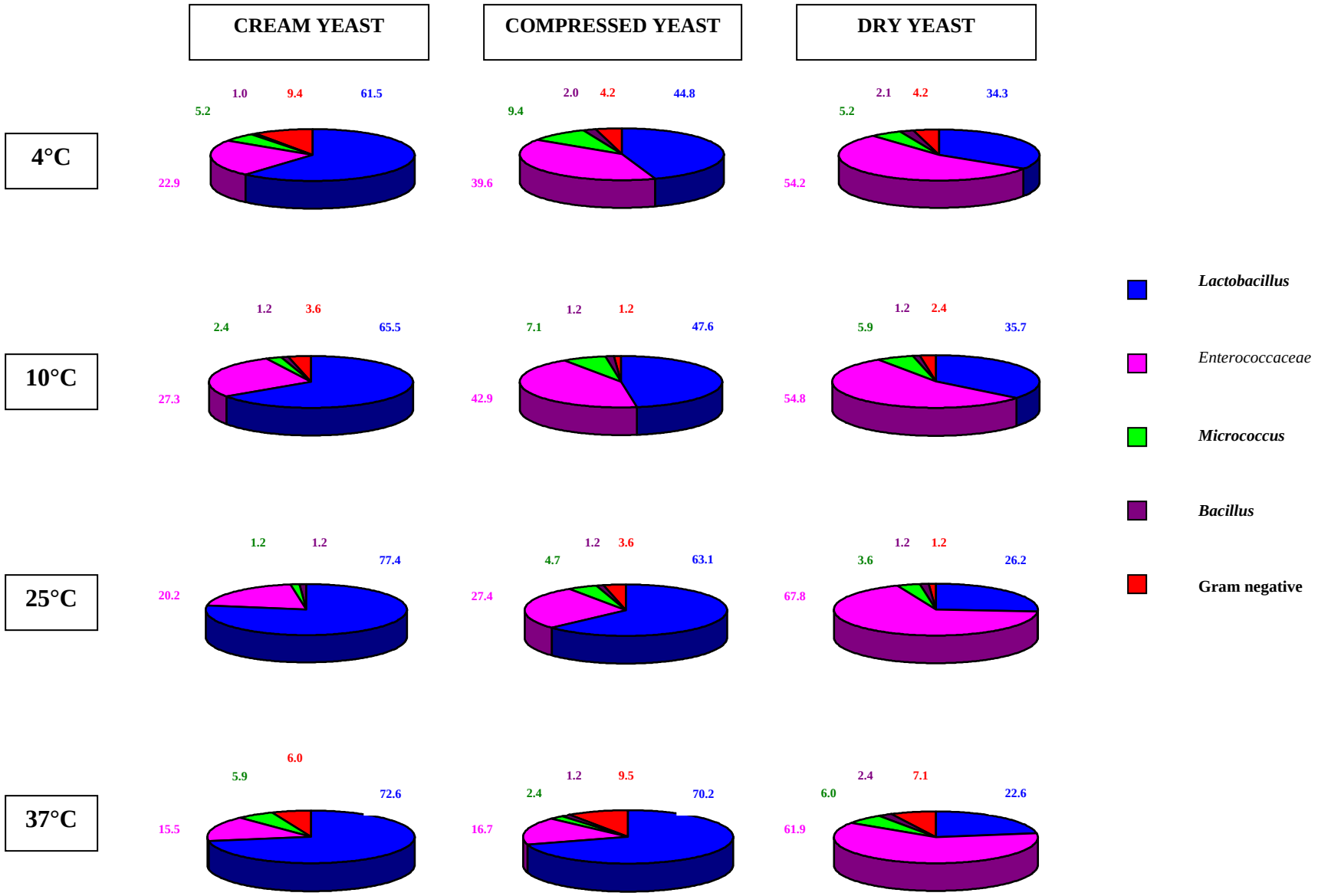


Figure 3.6 Overall percentage distribution of 1044 predominant bacteria isolated from aerobic plate counts of cream (348 isolates), compressed (348 isolates) and dry (348 isolates) yeast samples stored at 4, 10, 25 and 37°C for 21 days.

CHAPTER FOUR

MICROBIOLOGICAL SURVEY AND BIOFILMS ASSOCIATED WITH THE POST-FILTRATION ENVIRONMENT IN BAKER'S COMPRESSED YEAST PRODUCTION

ABSTRACT

Equipment surfaces and the processing environment associated with the production of Baker's compressed yeast were assessed for microbial biofilms. During three replicate studies, viable yeast and bacteria (APC, *Enterococcus*, coliforms) were enumerated from five processing equipment surfaces, found post-filtering, using stainless steel 'mock' surfaces and swabbing, after 7, 14, 21 and 28 days of yeast production. Viable yeast counts rarely exceeded 5 log CFU/ cm^2, which was equivalent to approximately 75% coverage on of all 'mock' surfaces. Counts of bacteria attached to 'mock' surfaces increased over 28 days. *Enterococcus* counts ranged from 2.30 log CFU/ cm^2 to 4.69 CFU/ cm^2, and aerobic plate counts (APC) ranged from 2.17 CFU/ cm^2 to 4.89 CFU/ cm^2. Equipment surfaces which consistently recorded the highest numbers of attached coliforms (*ca.* 2 log CFU/ cm^2), APC and *Enterococcus* (*ca.* 4.5 log CFU/ cm^2) were the hopper and extruder. Since counts obtained using the swab technique were only comparable with the 'mock' surface technique within 1 to 2.5 log CFU/ cm^2, it is recommended that swabbing be used as a semi-quantitative tool for estimating the cleanliness of yeast processing equipment surfaces. Scanning electron microscopy of 'mock' surfaces confirmed the accumulation of yeast cells with time and the presence of rod and coccoid-shaped bacterial cells. Characterization of 318 predominant isolates obtained by both the swab and 'mock' surface technique indicated the microbial populations associated with the equipment surfaces consisted of *Enterococcaceae* (70%), *Lactobacillus* (20%) and Gram-negative rods (10%). The attachment of various species of *Enterococcus*, specifically *E. avium* (28%) and *E. faecium* (20%), to yeast processing equipment surfaces was confirmed. Settle plate counts confirmed the presence of *Enterococcus*, coliforms and *E. coli* in the air adjacent to compressed yeast processing equipment (post-filtration), while hand swabs eliminated workers as significant sources of product contamination with *E. coli*, coliforms and *S. aureus*. Improved cleaning regimes, incorporating mechanical methods such as rubbing and scraping, should be considered applied for controlling biofilm formation on product contact surfaces.

INTRODUCTION

The attachment of microorganisms on processing equipment surfaces is of serious concern in food industries (Zottola and Sasahara, 1994; Chumkhunthod *et al*., 1998, Hood and Zottola, 1997; Kumar and Anand, 1998; Gibson *et al*., 1999), and are often potential sources of contamination for food products that may come into contact with these surfaces (Assanta *et al*., 2002). Food processing environments have an abundance of exposed surfaces on which microorganisms may attach, grow and develop into biofilms (Willcock *et al*., 2000). Biofilms are defined as a functional consortium of microorganisms attached to a surface embedded in the extracellular polymeric substances (EPS) produced by the microorganisms (Costerton *et al*., 1987; Pereira *et al*., 2002). The formation of a biofilm is a dynamic process and different mechanisms are involved in their attachment and growth (Kumar and Anand, 1998).

Biofilms can be formed on a variety of materials commonly used in food processing plants (Chumkhunthod *et al*., 1998), including stainless steel, aluminum, glass, Teflon seals and nylon materials (Notermans *et al*., 1991). Biofilm formation is initiated when microorganisms approach a nutrient conditioned surface (Costerton, 1995), transported either by diffusion, turbulent flow of liquids, or flagella (Kumar and Anand, 1998). This is followed by the attachment of microorganisms to the conditioned surface by reversible and irreversible adhesion. During reversible adhesion, weak long-range interactions, e.g. van der Waals attraction forces, electrostatic forces and hydrophobic interactions, develop between microorganisms and the surface (Kumar and Anand, 1998). During this stage of attachment, cells can be easily removed from the surface (Hood and Zottola, 1997). The production of surface attachment appendages by bacteria, such as fimbriae, pili and EPS fibrils, together with short-range forces, e.g. dipole-dipole interactions, hydrogen, ionic and covalent bonding, corresponds with the irreversible adhesion of microorganisms to a surface (Kumar and Anand, 1998). In this process, the removal of cells requires much stronger forces, such as scrubbing or scraping (Marshall *et al.*, 1971). The irreversibly attached cells grow and divide forming discrete microcolonies, which are the basic organizational sub-units of a biofilm (Costerton, 1995). The attached cells also produce EPS which anchors the cells to the surface (Marshall, 1992), and maintains the structural stability of the microcolonies (Characklis and Marshall, 1990). The continuous attachment of

microorganisms to the surface and subsequent growth along with EPS production forms a mature biofilm with highly permeable water channels (Costerton *et al*., 1994). As the biofilm ages, attached cells detach and disperse from the biofilm in order to survive and colonize new niches (Kumar and Anand, 1998).

It has been well documented that biofilm cells exhibit increased resistances to antimicrobial agents than their corresponding planktonic forms (Carpentier and Cerf, 1993; Frank and Chmielewski, 1997, Gilbert *et al.*, 1997; Chumkhunthod *et al*., 1998). Mechanisms which may attribute to this increased resistance include reduced diffusion, physiological changes due to reduced growth rates and the production of enzymes by biofilm cells capable of degrading antimicrobial substances (Kumar and Anand, 1998). In addition, the production of EPS during mature biofilm formation may also act as a physical barrier, molecular sieve and adsorbent of conventional sanitizers and antibiotics (Anwar *et al*., 1992; Boyd and Chakrabarty, 1995).

Microbial cells in biofilms may survive on processing equipment surfaces if the latter are not properly cleaned and sanitized (Chumkhunthod *et al*., 1998). The presence of biofilms on food processing surfaces can lead to hygienic problems and economic losses due to spoilage of product or foodborne disease (Carpentier and Cerf, 1993). In many food processing industries, a typical cleaning regime involves the removal of bulk product debris (soils), cleaning, sanitation and rinsing. During cleaning, the chemicals in a detergent break down food soils and reduce their attachment strength to facilitate their removal from the equipment surface, whilst chemical sanitizers reduce the viability of microorganisms remaining after cleaning (Gibson *et al*., 1999). There are several factors which aid in the efficient cleaning of processing equipment, for example mechanical cleaning (brushing, scrubbing, or cleaning-in-place), temperature, and contact time. Provided that the equipment and environment are hygienically designed, a good cleaning and sanitation program can be an effective method to control biofilm formation on processing equipment surfaces (Gibson *et al*., 1999). In the commercial production of yeast, microorganisms may grow as biofilms in various niches in fermenters, pipelines, or on processing equipment surfaces. Processing equipment is highly complex and often has inaccessible parts, which are often difficult to clean and sanitize effectively. Any deposits of yeast product on equipment surfaces can become a ready source of nutrients for subsequent bacterial attachment and biofilm

formation (van Dam, 1986; Reed and Nagodawithana, 1991). The continuous use of contaminated equipment could cause parts of the biofilm to detach and spread further in the processing line which may introduce a variety of spoilage (e.g. lactic acid bacteria) or pathogenic bacteria (e.g. *Listeria monocytogenes*) into the finished yeast product. This could result in subsequent spoilage of the commercial yeast product or health risks associated with use as a raw material in baking, brewing and wine making industries. Due to strongly increasing demands for microbiological quality and safety of commercially manufactured yeast, the prevention of biofilm formation on yeast processing equipment surfaces by consistent cleaning and sanitation is imperative.

Various techniques have been adopted for the study and understanding of biofilm attachment and control (Kumar and Anand, 1998). *In situ* studies of biofilms in food processing environments often have areas where biofilms can grow, which are difficult to access. Different methods in microscopy, especially scanning electron microscopy (SEM) of surfaces have gained considerable attention in the study of the adhesion of bacteria to inert surfaces (Lewis *et al.*, 1987) and biofilm formation (Notermans *et al*., 1991; Zottola, 1991). SEM has been used in previous *in situ* studies of biofilm formation on processing equipment using 'mock' surfaces, such as in poultry processing (Lindsay *et al*., 1996) and processed meat manufacturing (Kotze, 2002).

This *in situ* biofilm study is different from any other, in that Baker's yeast is a living product, which may promote bacterial biofilm formation on processing equipment surfaces. Previous studies of a commercial yeast factory have found Gram-positive cocci (*Enterococcus* spp.) and Gram-negative rods (*E. coli* and coliforms) in finished Baker's compressed yeast products (O'Brien *et al*., 2004a). It is thought that this contamination may partly originate from bacterial biofilms on processing equipment surfaces. This study therefore aimed to (i) quantify the attachment of yeast and bacteria on yeast processing equipment surfaces over time, (ii) visually examine biofilm formation on yeast processing equipment surfaces using SEM, (iii) determine the effect of yeast product build-up on the attachment of bacteria and biofilm formation and (iv) evaluate the potential contribution of vectors such as the air and workers to product contamination.

MATERIALS AND METHODS

Three independent replicate studies were conducted over 5 months, from January to May 2004 (Figure 4.1).

(a) Sampling of yeast processing equipment surfaces for attached microbial populations

Stainless steel 'mock' surfaces (2 x 2 cm, 0.5 mm thick, grade 304L) were used to assess *in situ* biofilm formation on stainless steel processing equipment used in the manufacture of Baker's compressed yeast (PAT), according to Lindsay *et al*. (1996). Following the filtration of yeast cream into yeast cake, the product is no longer in a closed-system, but susceptible to the surrounding processing environment, and was therefore used as a model system for attachment studies. In addition, the processing equipment located downstream of the filter-press, was chosen for ease of sampling of 'mock' surfaces. Prior to use, stainless steel 'mock' surfaces were degreased in acetone for 1 min, soaked in 70% ethanol for 1 min, flamed and sterilized by autoclaving. Eight 'mock' surfaces were attached aseptically to five processing equipment surfaces: filter hopper (A), filter conveyor line for filtered yeast cake (B), PAT hopper (C), PAT extruder (D) and the PAT conveyor line for 5kg blocks of compressed yeast prior to packaging (E), using silicon glue (Universal 1001, Oosterhout, Holland) (Figure 4.2). After allowing the glue to dry for 10 minutes, the exposed 'mock' surface was swabbed with 70% ethanol to remove any incidental contamination that may have occurred during the attachment procedure (Lindsay *et al*., 1996).

On each sampling occasion 'mock' surfaces were removed after standard factory cleaning regimes. Using sterile forceps, duplicate 'mock' surfaces were aseptically removed from each processing site, after 7, 14, 21 and 28 days. The side of the stainless steel 'mock' surface facing the silicone glue was marked by scratching with a sterile blade, and the glue peeled off aseptically (Lindsay *et al*., 1996). Surfaces were placed directly into individually labeled sterile 100 ml Schott bottles and kept on ice during transportation to the laboratory for analysis on the same day. For each analysis, unused 'mock' surfaces served as negative controls.

(b) Swabbing of yeast processing equipment surfaces and workers' hands

Coinciding with the removal of stainless steel 'mock' surfaces after 7, 14, 21 and 28 days, each processing site was swabbed within 10 cm of the removed 'mock' surface. On the day of sampling, a pre-autoclaved swabbing template (4 x 2 cm) was degreased with acetone and 70% alcohol for 1 minute and positioned on the sampling site. Following the manufacturer's instructions, the 3M Quick Swab (3M, St. Paul, USA) was rubbed thoroughly over the sampling site within the positioned template for 30 seconds. In addition, on each sampling occasion, bacterial numbers present on the hands of three workers (2 filter press operators and 1 PAT packing line operator) were quantified by plate counting, as well as for the presence of *Staphylococcus* (*S.*) *aureus* using duplicate 3M™ Petrifilm™ Rapid *S. aureus* count plates (3M) (Chapter 2.2). Workers' hands were swabbed for 30 seconds using the 3M Quick Swab (3M). All swabs were kept on ice during transportation to the laboratory and analysed within 24h.

(c) Preparation and microbiological analysis of samples

All 'mock' surfaces were individually rinsed with sterile distilled water for 30 seconds. For duplicate surfaces, one surface was prepared for scanning electron microscopy (SEM), while the other was prepared for microbiological analysis (Lindsay *et al*., 1996). For microbiological analysis, attached yeast and bacteria were removed from each stainless steel sampling surface by shaking for 20 min in 20 ml sterile saline solution supplemented with (0.1%) saline and 20 g glass beads (0.5 mm in diameter) (Unitek, Johannesburg, South Africa) as described by Lindsay *et al.* (1996). Following dislodging, the solutions were allowed to stand for 1 h at room temperature (*ca*. 25°C), permitting recovery of damaged cells before analysis (Lindsay *et al.*, 1996).

Single tenfold dilutions in sterile buffered peptone water (BPW) (Oxoid, Basingstoke, U.K) were prepared for all sample types and plated in duplicate using standard pour-plate techniques and KF Streptococcus Agar (Oxoid) (*Enterococcus* count), Rapid' *E. coli* 2 Agar (Bio-rad, Marnes-La-Coquette, France) (Coliform and *E. coli* count), spread-plate techniques and WL Nutrient Agar (Oxoid) (Aerobic plate count) and Malt Extract Agar (MEA) (acidified with 10% Lactic acid, pH

3.5) (Oxoid) (viable yeast count). All plates were incubated and counted as described previously (Chapter 2.1, Table 2.1.2). Viable yeast count plates were incubated at 30°C for 48 h (Figure 4.3). After incubation, plates showing between 30 and 300 colonies (or the highest number if below 30) were counted and the log colony forming units (CFU) per cm^2 ('mock' surfaces and equipment surface swabs) or per hand (workers' hand swabs) determined.

(d) Bacteriological analysis of air inside the post-filtration processing area

On each sampling occasion, 7, 14, 21 and 28 days, bacterial numbers of the air immediately adjacent to the five processing areas were quantified by duplicate settle plates (standard 90 mm Petri dishes) containing the same media (Chapter 2.1, Table 2.1.2) over an exposure time of 5 hours (morning shift). Processing areas for settle plate analysis are shown in Figure 4.4. Plates were incubated immediately (Chapter 2.1, Table 2.1.2), and averaged counts reported as CFU per plate.

(e) Statistical analysis

Counts obtained for each sample type for the three replicate studies were meaned and standard deviations between samples calculated. Standard deviations between the same sample types were calculated in order to assess the variability between the three studies. In addition, colony count data were statistically analysed using the analysis of variance (ANOVA) test at the 95% confidence level using the STATGRAPHICS (STSC Inc. and Statistical Graphics Corporation) programme.

(f) Preparation of stainless steel 'mock' surface samples for SEM

For scanning electron microscopy, stainless steel 'mock' surfaces were fixed in 3% aqueous gluteraldehyde overnight at room temperature and dehydrated in an ethanol series (20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 96%, 100% twice) for 10 min in each concentration at room temperature (*ca.* 25°C). Samples were then subject to critical point drying, mounted, coated with carbon and gold-palladium (10nm) for 10 min and viewed on a JSM-840 SEM (Lindsay *et al.*,

1996). Unused sterile stainless steel 'mock' surfaces were prepared in the same way and served as controls.

(g) Characterization of predominant bacterial populations associated with yeast processing equipment surfaces

Bacterial populations were isolated from duplicate WL Nutrient agar (Oxoid, Basingstoke, UK) + 1% cyclohexamide (0.004g/ litre) (Scharlau, Barcelona, Spain) total aerobic plate count plates, using plates of highest dilutions showing growth (von Holy and Holzapfel, 1988), for each stainless steel 'mock' surface and equipment swab sample. In this way, a total of 318 isolates were obtained for 'mock' surface (numbering 180) and equipment swab (138) samples. Bacterial isolates were purified and characterized to genus level using the dichotomous key of Fisher *et al.* (1986) as described in Chapter 2.2. In addition, 176 presumptive *Enterococcus* isolates were obtained from KF Streptococcus agar plates (Oxoid) of stainless steel 'mock' surface samples and purified. Isolates which were Gram-positive, catalase negative cocci, capable of growth on bile esculin agar (Oxoid) were further characterized using the BBL CRYSTAL™ Gram-Positive Identification system (Becton, Dickinson and Company, USA) (Figure 4.5).

RESULTS

(a) Bacterial and yeast counts on stainless steel 'mock' surfaces and swabs of yeast product contact surfaces

For all processing equipment surfaces, the highest aerobic plate counts (APC) were generally obtained after 28 days, followed by those at 21, 7 and 14 days, in decreasing order (Figure 4.6). In general, *Enterococcus* counts increased with time, being the lowest after 7 days, followed by those taken at 14, 21 and 28 days, in increasing order. After 7 days, levels of attached yeast (5.49 log CFU/ cm^2), *Enterococcus* (3.45 log CFU/ cm^2) and APC (3.66 log CFU/ cm^2) were highest on 'mock' surfaces attached to the PAT hopper, and lowest on the on 'mock' surfaces attached to the filter hopper (*ca.* 2 log CFU/ cm^2 APC and *Enterococcus*, 4.5 log CFU/ cm^2 viable yeast) (Figure 4.6, 1A and 1C). After 28 days, the highest bacterial counts were recorded on two surfaces, the

PAT hopper and PAT extruder, which exhibited APC of 4.89 log CFU/ cm^2 and 4.57 log CFU/ cm^2, respectively. These two surfaces also exhibited the highest *Enterococcus* counts which progressively increased over the 28 day sampling period reaching 4.69 log CFU/ cm^2 and 4.36 log CFU/ cm^2 (Figure 4.6, 1C and 1D). The highest number of attached yeast cells were also recorded for 'mock' surfaces attached to the PAT hopper after 7 days (5.49 log CFU/ cm^2) and the PAT conveyor after 14 days (5.18 log CFU/ cm^2). For all other surfaces, yeast counts did not exceed 5 log CFU/ cm^2 (Figure 4.6). Coliform attachment was recorded for three surfaces (i.e. filter conveyor, PAT hopper and PAT extruder), reaching *ca.* 2 log CFU/ cm^2 after 28 days (Figure 4.6, 1B, 1C and 1D). The attachment of *E. coli* (1.47 log CFU/ cm^2) was only observed on 'mock' surfaces attached to the PAT extruder after 28 days (Figure 4.6, 1D).

For most of the processing equipment surfaces, the number of bacterial and viable yeast cells retrieved using the swab technique were significantly lower ($P < 0.05$) than those obtained with the 'mock' surface and dislodging procedure, however results were generally comparable within 1 to 2.5 log CFU/ cm^2. Similarly to the 'mock' surface results, the highest number of attached viable yeast (3.88 log CFU/ cm^2), *Enterococcus* (2.62 log CFU/ cm^2) and APC (2.65 log CFU/ cm^2) were recorded for swabs of the PAT hopper, whilst the lowest were obtained from swabs of the filter conveyor (0.73 log CFU/ cm^2 APC and *Enterococcus*, 1.1 log CFU/ cm^2 viable yeast) (Figure 4.6, 2A and 2C). Swabbing enumerated coliforms from two processing areas, the PAT extruder after 14 days (1.14 log CFU/ cm^2) and the PAT hopper after 28 days (0.83 log CFU/ cm^2). No *E.coli* was enumerated from swabs of the five processing equipment surfaces over the 28 day sampling period.

(b) Scanning electron microscopy of stainless steel 'mock' surfaces

Scanning electron micrographs of an uncolonised control surface are shown in Figure 4.7. The control surface is clearly marked by grooves and crevices characteristic of stainless steel, and showing the absence of attached yeast and bacterial cells. Compared to control surfaces, micrographs of stainless steel 'mock' surfaces taken from the filter hopper (Figure 4.8), filter conveyor (Figure 4.9), PAT hopper (Figure 4.10), PAT extruder (Figure 4.11) and PAT conveyor

line (Figure 4.12) after 7, 14, 21 and 28 days, illustrated the attachment of yeast and bacterial cells.

After 7 days, scanning electron micrographs of the filter hopper surfaces showed the attachment of yeast cells as well as single cell attachment of coccoid- and rod-shaped bacteria (Figure 4.8). After 21 days, images showed increased entrapment and attachment of bacterial cells within the grooves and crevices of the stainless steel surface, and the detachment of yeast and bacterial cells after 28 days (Figure 4.6 and Figure 4.8).

High numbers of attached yeast cells was observed for filter conveyor 'mock' surfaces after 7 and 14 days (Figure 4.9). After 14 days, the attachment of single rod-shaped bacteria was observed, as well as the presence of budding yeast cells forming short chains. Micrographs of the filter conveyor surfaces demonstrated the lowest numbers of attached yeast and bacterial cells after 21 and 28 days (Figure 4.9).

For PAT hopper and extruder 'mock' surfaces, Figure 4.10 and Figure 4.11 show that the number of bacterial cells attached to the stainless steel 'mock' surface were greater after 7 days than on any other processing surface area. The formation of microcolonies by rod-shaped bacteria can be seen on both surfaces after 7 days (Figure 4.10 and Figure 4.11). Micrographs of the PAT hopper and extruder surfaces after 7 days, also illustrated the highest numbers of attached yeast cells, forming confluent layers over the 28 day sampling period (Figure 4.10 and Figure 4.11). Micrographs of the PAT hopper surface showed surface features similar to imprints (footprints) of attached yeast cells and EPS formation between rod-shaped bacterial cells (Figure 4.10, day 14). After 21 days, micrographs of the PAT hopper surfaces revealed the attachment of rod-shaped bacteria to yeast cells, and after 28 days the increased attachment of coccoid-shaped bacteria to the attached yeast cells (Figure 4.10, day 28). For PAT extruder surfaces, the attachment of short and long rod-shaped bacteria predominated over the 28 day sampling period (Figure 4.11), attaching themselves to the yeast cells after 21 days.

After 7 days of exposure, micrographs of the PAT conveyor 'mock' surfaces also showed high numbers of attached yeast cells to the stainless steel, which over the 28 day sampling period

formed a layer covering most of the surface (Figure 4.12). The attachment of single coccoid- and rod-shaped cells was observed after 7 and 14 days, respectively. Similarly to the PAT hopper and extruder surfaces, the entrapment of rod-shaped bacteria by attached yeast cells was observed after 28 days.

(c) Bacterial counts associated with the air inside the processing area and workers' hands

Settle plate counts demonstrated the presence of all groups of bacteria in the air adjacent to the processing equipment used in the commercial manufacturing of Baker's compressed yeast (Figure 4.13). In all instances, levels of aerobic bacteria were higher than *Enterococcus*, coliforms and *E.coli*, respectively. The air adjacent to the end of the PAT packing line recovered the highest level of coliform organisms, reaching *ca*. 30 CFU/ plate, and was the only processing site where *E. coli* was detected (Figure 4.13, processing area 3E). The air directly adjacent to the filter conveyor line contained the highest levels of *Enterococcus* and corresponding aerobic bacteria, reaching *ca.* 55 and 90 CFU/ plate, respectively (Figure 4.13, processing area 3A). The air above the filter conveyor line/ PAT hopper contained the lowest numbers of *Enterococcus* and aerobic bacteria (Figure 4.13, processing area 3B), whilst the air surrounding the start of the PAT packing line contained the lowest number of coliform organisms (Figure 4.13, processing area 3D).

Corresponding with the sampling intervals for 'mock' surfaces and equipment swabs, the hands of processing operators were swabbed. The bacterial numbers present on the hands of three workers (2 filter press operators and 1 PAT packing line operator) are shown in Figure 4.14. The number of aerobic bacteria and *Enterococcus* on the hands of both filter press operators were *ca.* 1 log CFU/ hand higher than those obtained from the PAT packing line operator. Coliform organisms were only recovered from the hands of the filter-press operators reaching a maximum of 1 log CFU/ hand over the four-week sampling period. *E. coli* was not detected on any of the workers' hands, whilst *S. aureus* was detected on the hands of filter press operator 2 (25% of sampling occasions) and the PAT packing line operator 1 (17% of sampling occasions).

(d) Predominant bacterial populations associated with yeast processing equipment surfaces

The percentage distribution of bacterial populations isolated from stainless steel 'mock' surface and equipment swab samples is shown in Table 4.1. Gram-positive bacteria predominated on all 'mock' surface and equipment swab samples (73 to 100%; Table 4.1). Cells of the Gram-positive bacterial genus predominantly recovered belonged to *Enterococcaceae* (includes members of the genera *Enterococcus*, *Lactococcus*, *Leuconostoc* and *Pediococcus*), with total proportions ranging from 54 to 78% for 'mock' surfaces and 44 to 84% for equipment swabs (Table 4.1). The other predominant Gram-positive genus isolated from samples was *Lactobacillus*, with total proportions ranging from 8 to 31% for 'mock' surfaces and 11 to 31% for equipment swabs (Table 4.1). For 'mock' surfaces, higher proportions of *Enterococcus* were obtained after 21 days of exposure, while proportions of *Lactobacillus* were greater for day 7 and 14 samples. For 'mock' surfaces, the highest proportion of *Enterococcus* was isolated from the filter hopper (Table 4.1; 1A) and *Lactobacillus* from the PAT conveyor (Table 4.1; 1E). The highest proportion of Gram-negative rods were obtained from equipment swabs and 'mock' surfaces of the PAT hopper (16 and 23%; Table 4.1) and PAT extruder (25 and 27%; Table 4.1). A noteworthy finding was the similarity between the total populations isolated from the 'mock' surface and equipment swab samples, both comprising 70% *Enterococcus*, 20% *Lactobacillus* and 10% Gram-negative rods (Table 4.1).

Of the 176 presumptive *Enterococcus* isolates from stainless steel 'mock' surfaces, 69 (39%) were Gram-positive, catalase-negative cocci and capable of growth on Bile esculin agar. These isolates were further characterized using the BBL CRYSTAL™ Gram-Positive Identification system as *Ent. avium* (28%), *Ent. faecium* (20%), *Ent. casseliflavus* (16%), *Ent. durans* (10%), *Ent. faecalis* (7%), *Ent. hirae* (7%) and *Ent. raffinosus* (7%).

DISCUSSION

It is well documented that biofilm formation on product contact surfaces may contaminate the product directly, as the product touching or passing over the surface will potentially pick up

microbial contamination (Gibson *et al*., 1999). It is speculated that bacterial biofilms on processing equipment surfaces in the yeast factory could contribute to increased bacterial counts associated with Baker's compressed yeast product. After 7 days of compressed yeast production, the number of attached viable yeast cells already exceeded 4 log CFU/ cm^2 on all five processing equipment surfaces. However, over the 28 day sampling period, attached yeast counts rarely exceeded 5 log CFU/ cm^2. According to literature, *Saccharomyces* (*S*.) *cerevisiae* is a spherical to oval unicellular yeast ranging in size from 9 to 25 μm^2 (Prescott *et al.*, 1996). Using these measurements, the maximum coverage of a $1cm^2$ stainless steel surface by a single layer of *S. cerevisiae* cells, would be 6.6 log CFU/ cm^2, indicating the process equipment surfaces investigated in this study were approximately 75% occupied. This finding suggested a high level of product build-up on all processing equipment surfaces, indicating the inefficiency of current cleaning methods for the removal of product debris, which may be irreversibly attached to the stainless steel surfaces, and required more mechanical methods of removal.

In the food processing environment, the increased level of nutrients remaining on food-contact surfaces acts as a conditioning film for biofilm formation (Hood and Zottola, 1997). The build-up of yeast product not only conditioned the surface with nutrients for bacterial attachment, but according to our results, may have increased their entrapment and ability to form biofilms, since higher yeast counts correlated with increased attached bacterial counts in this study. For most stainless steel 'mock' surfaces, *Enterococcus* and aerobic counts were higher after 28 days of exposure than on any other day, exemplifying the ability of bacterial cells in biofilms to escape removal by standard cleaning regimes. This was also true for the attachment of coliform organisms and *E. coli* to processing equipment surfaces, which was only observed in the areas less accessible for cleaning, such as the PAT hopper and PAT extruder. These surfaces also showed the highest number of attached *Enterococcus* cells and aerobic bacteria (*ca*. 4 to 5 log CFU/ cm^2), in most cases equating the number of viable yeast cells attached to the surface. Therefore, in addition to being difficult processing areas to clean, the PAT hopper and extruder, may also provide 'moistened' protected niches for bacterial attachment and biofilm formation during processing, and should be the focal points for improved cleaning.

The swabbing of processing equipment surfaces, confirmed the attachment of yeast and bacterial cells to the stainless steel processing equipment surfaces found in the yeast factory. However, the counts obtained from swabs of the equipment surfaces were significantly lower ($P < 0.05$) than those obtained using the 'mock' surface and dislodging technique. Reportedly, the enumeration of microorganisms from surfaces using traditional hygiene swabbing is greatly influenced by a number of factors, such as the ability to remove bacteria from a surface; the release of bacteria from the swab after sampling; and the overall recovery of injured cells (Moore and Griffith, 2002). In addition, our results showed that swabbing may not remove bacterial cells entrapped within the grooves and crevices of the stainless steel, the latter viewed using scanning electron microscopy. The difference between counts obtained using the swab and 'mock' surface technique were greater for yeast cells than bacteria, suggesting that the yeast cells had irreversibly attached to the stainless steel surfaces and were not as a result of simply product residue on the processing equipment surfaces. Therefore, irreversibly attached yeast and bacterial cells would require mechanical forces, such as those used in the dislodging technique, for the total enumeration of attached cells.

Common areas for biofilm formation in food-processing environments reportedly include the floors, bends in pipes, rubber seals, conveyor belts, and stainless steel surfaces (Hough *et al*., 1982; Kumar and Anand, 1998). Scanning electron images confirmed the attachment of yeast cells to stainless steel equipment surfaces, as well as the entrapment and attachment of bacterial cells. A range of bacterial morphologies were encountered, ranging from cocci to rods. However, the predominant cell morphology on all processing equipment surfaces was rod-shaped. Reportedly, contact with a surface such as stainless steel may trigger a mechanism for the production of EPS (Hood and Zottola, 1997). Reportedly, high quantities of exopolymers are not required for the first step in biofilm formation but are needed to develop a true biofilm matrix. EPS production by attached bacteria was not visible in the majority of micrographs viewed in this study. In general, the development of bacterial biofilm on stainless steel surfaces was rare, in most instances single cell attachment was observed with early stages of microcolony formation. This was also true for attached yeast cells, which after 28 days showed little evidence of EPS production. The most documented biofilm formation by yeast is *Candidia* (*C.*) *albicans* on clinically relevant surfaces such as bioprosthetic materials and urinary catheters. A study by

Chandra *et al.* (2001), found the ability of *C. albicans* to form biofilms contrasted with that of *S. cerevisiae*, which failed to form EPS and mature biofilm.

Interactions between various microbial populations during the initial stages of biofilm formation, has a significant effect on the ultimate structure and physiology of the biofilm (James *et al*., 1995). It is well documented that in mixed species biofilms, the EPS produced by one species may enhance the stability of other species within a biofilm (McEldowney and Fletcher, 1987; Kumar and Anand, 1998). In our study, higher bacterial counts correlated with higher yeast counts. However, micrographs showed little evidence of biofilm formation occurring between yeast and bacterial cells, suggesting the yeast cells conditioned the surface for attachment and entrapment of bacterial cells, without forming a binary species biofilm.

In many instances bacterial and yeast counts fluctuated over the 28 days of processing. This was also true for scanning electron micrographs, which showed surface features suggested to be the imprints (footprints) of yeast cells and varying numbers of attached yeast and bacterial cells throughout the 28 day sampling period. These results suggested that the removal of attached cells may have occurred during cleaning, or that attached cells may have detached themselves from the stainless steel surface during processing. The ability of bacteria attached to surfaces as biofilms to transfer to new surfaces is not only imperative for long-term survival within a manufacturing plant, but is also central to the problems of product contamination (Willcock *et al*., 2000; Assanta *et al*., 2002). Cleaning is thought to be the most important stage for minimizing microbial colonization and removing attached microorganisms (Dunsmore, 1981; Carpentier and Cerf, 1993). The time available for biofilm formation is dependent upon the frequency of cleaning (Gibson *et al*., 1999). Often there is more time available for the formation of biofilms on environmental surfaces than processing equipment surfaces, since the former is usually cleaned on a weekly basis, whilst processing surfaces are cleaned several times a day (Gibson *et al*., 1999). In this study, even after cleaning, stainless steel processing equipment surfaces were visibly covered by a layer of yeast product residue. This finding, in addition to the images obtained using scanning electron microscopy, suggested that current cleaning practices are not efficient on either a microscopic or macroscopic level.

Reportedly, environmental surfaces such as floors and walls may be indirect sources of microbial contamination that can be transferred to the product by vectors such as the air and workers (Gibson *et al*., 1999). In this study, settle plate counts confirmed the presence of *Enterococcus*, coliforms and *E. coli*, bacterial species previously associated with finished compressed yeast product (Chapter 2.1) (O'Brien *et al.*, 2004a), in the air adjacent to yeast processing equipment. These results suggested, air movement around the processing area, could contribute to the bacterial contamination of commercially manufactured yeast. This is not the first time the environment has been highlighted as a potential source of product contamination. For example, a study by Dykes *et al*. (1991), found high levels of Enterobacteriaceae, yeasts and LAB in the air adjacent to the packing tables used in the manufacturing of smoked vienna sausages. In our study, the highest numbers of coliform organisms were found in the air adjacent to the packing line. It is suggested that the manufacturer install an enclosure around the open processing areas, which would prevent the settling of airborne contaminants on the surface of compressed yeast cakes. The contribution of workers' hands as sources of product contamination was negligible with respect to *E. coli* and coliform organisms. Low numbers of aerobic bacteria and *Enterococcus* could, however, contribute to the bacterial contamination of compressed yeast if handled during processing. The incidence of *S. aureus* on workers' hands was low, detected in 14% of all hand swabs. Although the counts associated with the workers' hands were low in this study, as a precautionary measure hygiene training of all workers is suggested together with more consistent hand washing, if workers are to handle passing product.

In our study, the bacterial populations recovered from 'mock' surface and equipment swab samples were similar to the populations previously associated with finished compressed yeast product samples (Chapter 2.2). This finding suggested that *Enterococcaceae*, *Lactobacillus* and Gram-negative rods associated with compressed yeast product may accumulate on processing equipment surfaces forming biofilms, and later acting as a continuous source of contamination between batches. The predominance of Gram-positive coccoid-shaped *Enterococcaceae*, did not correlate with the micrographs observed in this study, which showed the predominance of rod-shaped bacteria on most surfaces. This discrepancy could be explained by the smaller size of coccoid-shaped bacteria, resulting in their reduced visibility within the crevices of stainless steel or by their entrapment beneath layers of yeast cells. The weekly recovery of bacterial populations

from ‘mock’ surfaces indicated that *Lactobacillus* may be the primary colonizer of stainless steel, being isolated after 14 and 21 days of exposure, whilst *Enterococcaceae* were predominantly isolated after 28 days. Gram-negative rod-shaped bacteria were predominantly isolated from the PAT hopper and PAT extruder, reconfirming the attached coliform counts associated with the ‘mock’ surfaces and equipment swab samples (*ca.* 1 - 2 log CFU/ cm^2). Reportedly, enterococci are able to survive for long periods of time on a variety of inanimate objects (Bale *et al*., 1993). A study by Gelsomino *et al.* (2002), demonstrated how enterococci survived and grew in the inaccessible corners of a milking machine and bulk storage tank, directly contaminating the milk used in the manufacture of raw-milk cheese. Previous findings have suggested commercial yeast processing equipment as reservoirs of enterococci and direct sources of product contamination (Chapter 2.2) (O’Brien *et al*., 2004a). In this study, the attachment of various species of *Enterococcus* to stainless steel yeast processing equipment surfaces was confirmed. In a previous study by Viljoen and von Holy (1997), 50% of all isolates obtained from equipment surfaces associated with a brown bread production line were identified as *Enterococcus* spp.

CONCLUSION

Results from this study documented yeast product build-up and bacterial attachment on stainless steel processing equipment used in the manufacturing of Baker’s compressed yeast. The rough topography of the stainless steel processing equipment surfaces, together with the continuous attachment of yeast cells, entrapped bacterial cells. It is therefore suggested that a consistent and thorough cleaning program be implemented, especially in the less accessible processing areas, preventing equipment as continuous sources of bacterial contamination. Improved cleaning regimes need to evaluate the time intervals between cleaning and sanitation, efficacy of cleaning, type and concentration of detergent and sanitizer, and temperature upon application. Although swabbing only enumerated the transient bacteria associated with equipment surfaces, it is still a reliable and inexpensive technique for the continual assessment of yeast and bacterial counts associated with yeast processing equipment surfaces.

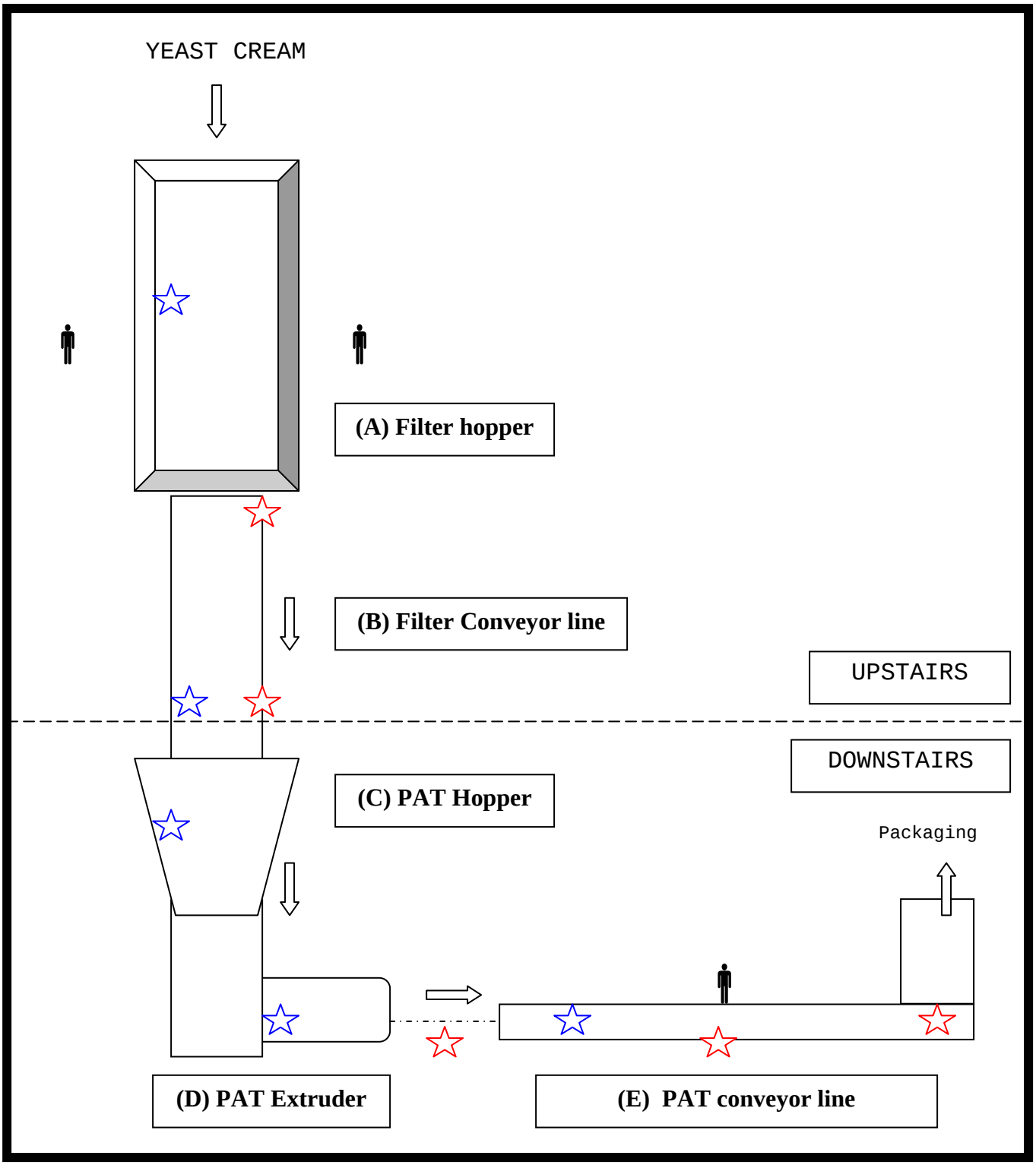
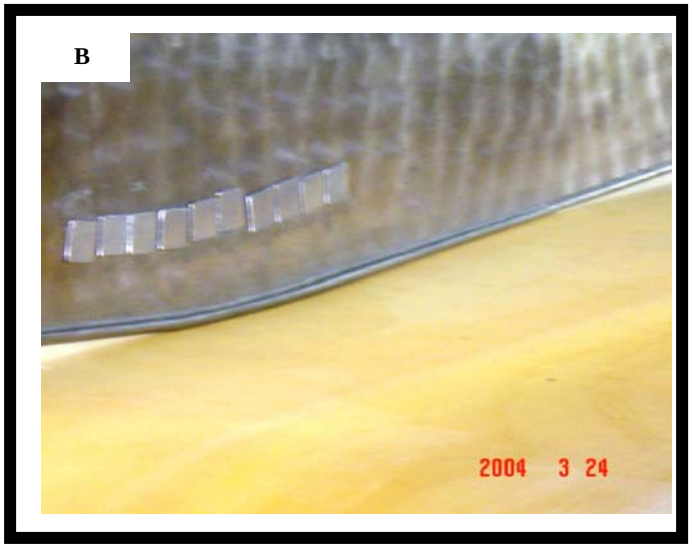
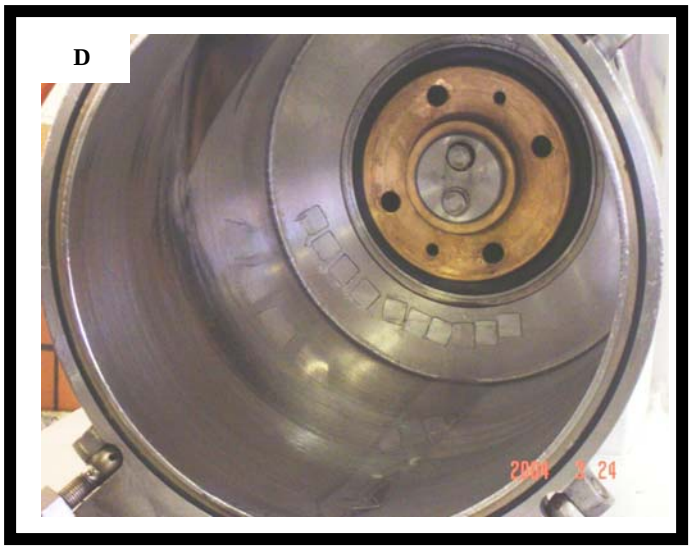


Figure 4.1 Floor plan of the Baker's compressed yeast manufacturing process with sampling areas **(A)** Filter hopper, **(B)** Filter conveyor line, **(C)** PAT hopper, **(D)** PAT extruder and **(E)** PAT conveyor line. Sample areas for stainless steel 'mock' surfaces and process equipment swabs (☆), hand swabs (🧍), and settle plates (☆).





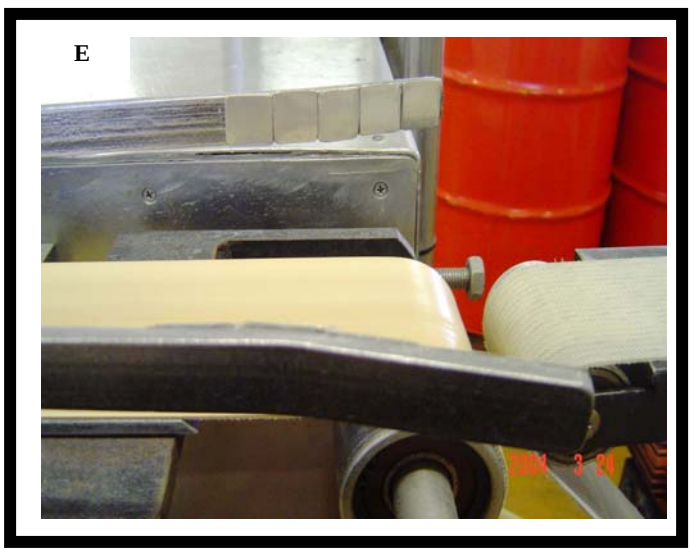


Figure 4.2 Stainless steel 'mock' surfaces attached to **(1A)** Filter hopper, **(1B)** Filter conveyor line for filtered yeast cake, **(1C)** PAT hopper, **(1D)** PAT extruder and **(1E)** PAT conveyor line for 5kg blocks of compressed yeast prior to packaging. The mock surfaces were aseptically attached to the processing equipment using silicone glue (Universal 1001), and removed after 7, 14, 21 and 28 days.

Figure 4.3 Malt Extract Agar (MEA) acidified with 10% Lactic acid, pH 3.5 (Oxoid), was used to determine viable yeast counts associated with stainless steel mock surface and equipment swab samples.

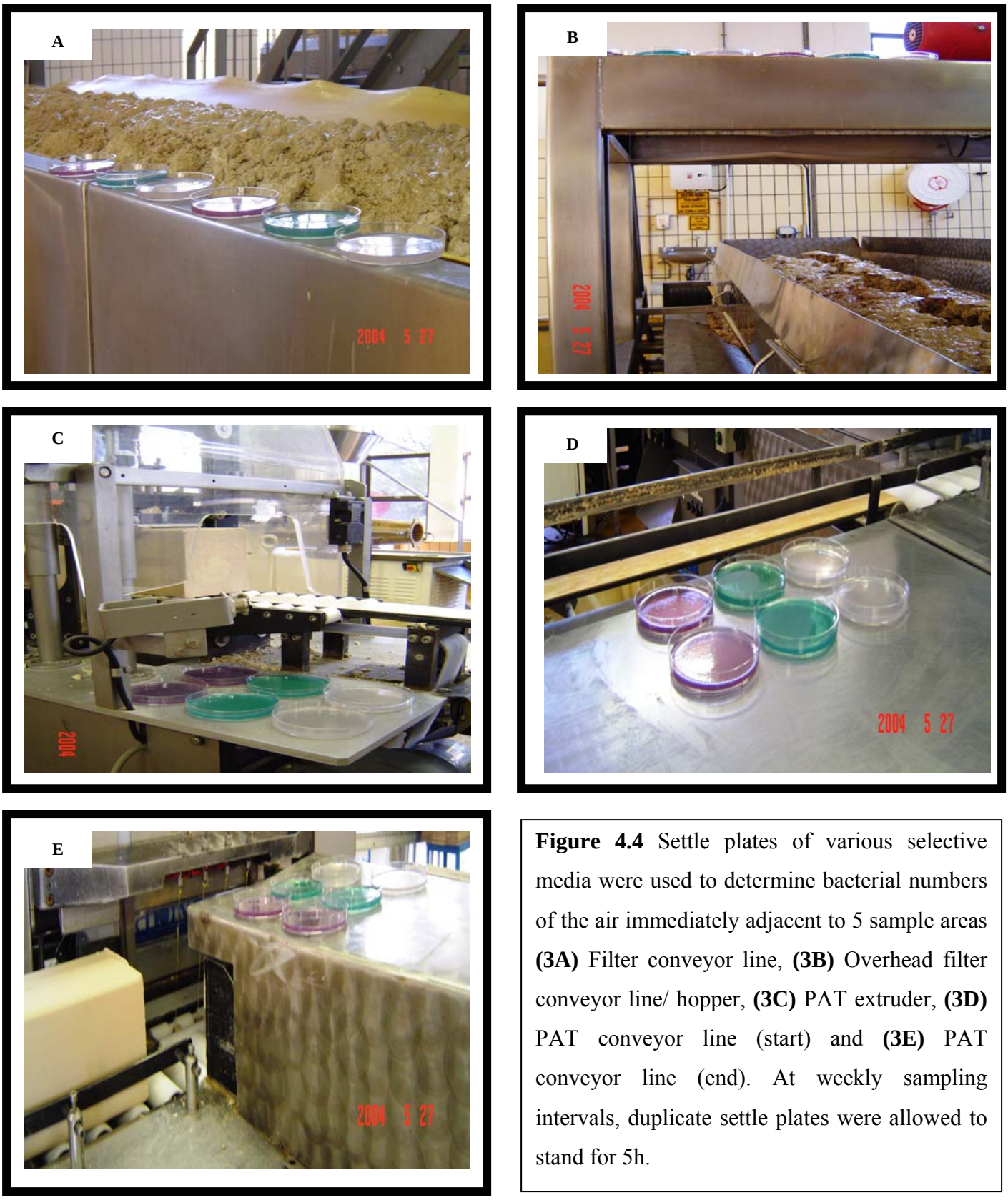


Figure 4.4 Settle plates of various selective media were used to determine bacterial numbers of the air immediately adjacent to 5 sample areas **(3A)** Filter conveyor line, **(3B)** Overhead filter conveyor line/ hopper, **(3C)** PAT extruder, **(3D)** PAT conveyor line (start) and **(3E)** PAT conveyor line (end). At weekly sampling intervals, duplicate settle plates were allowed to stand for 5h.

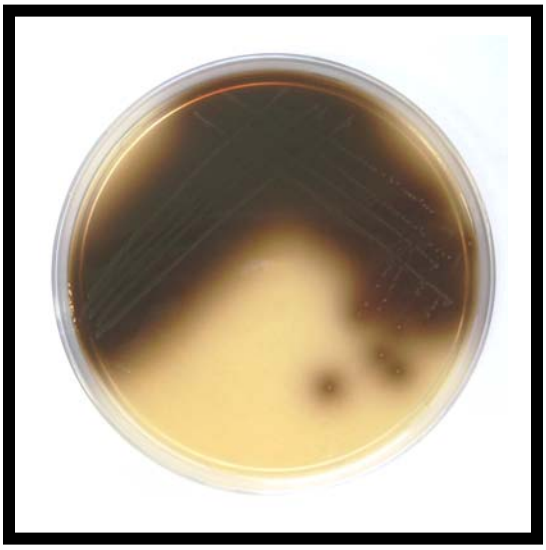


Figure 4.5 The differential medium, Bile Esculin Agar (BEA) (Oxoid), was used for the presumptive identification of enterococci, whose assimilation of esculin during growth blackens the media.

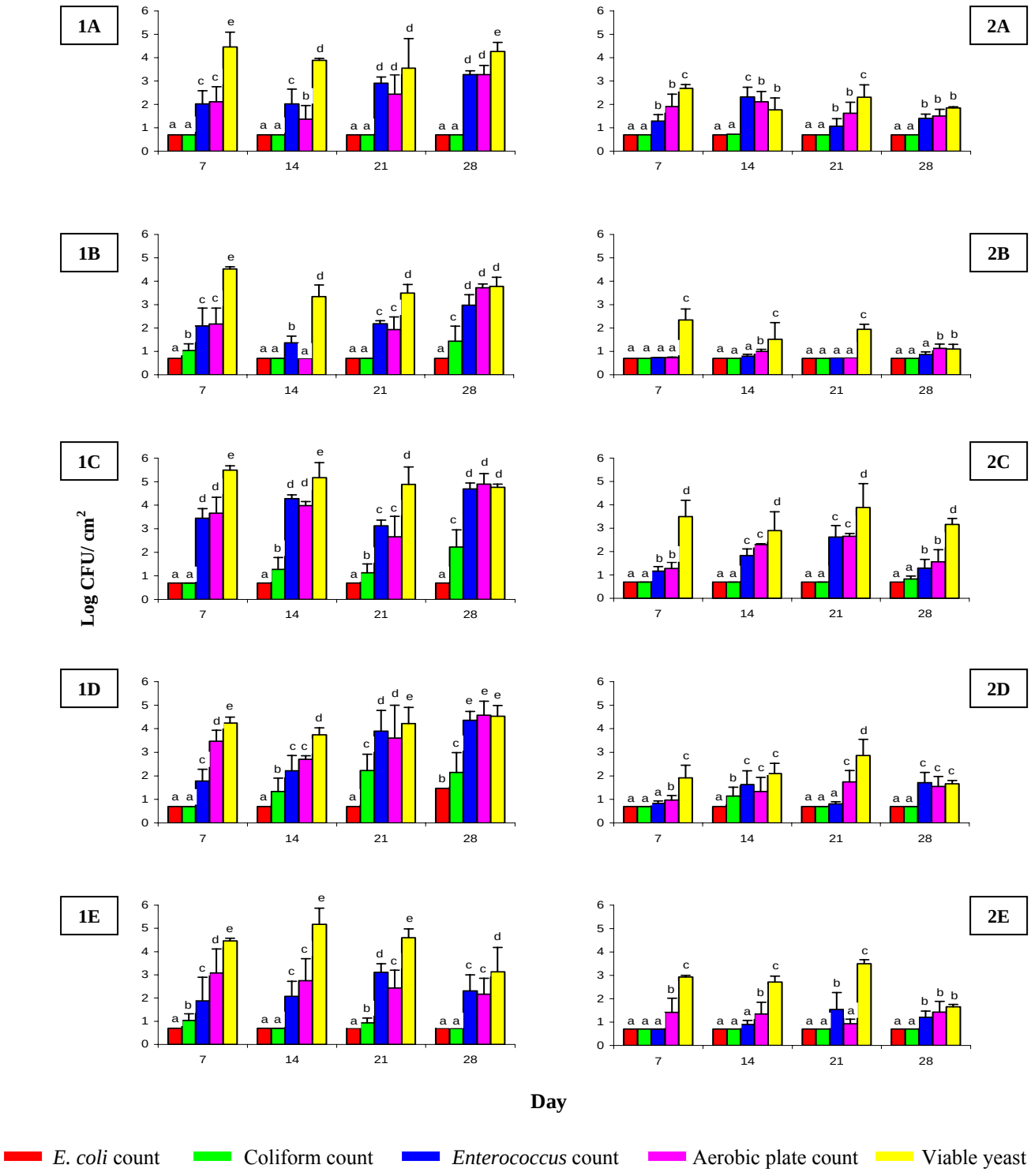


Figure 4.6 Mean numbers of *E. coli*, coliforms, *Enterococcus* and aerobic bacteria on five stainless steel mock surfaces **(1A to 1E)** and corresponding surface swabs **(2A to 2E)** for five processing equipment surfaces **(A)** Filter hopper, **(B)** Filter conveyor line, **(C)** PAT hopper, **(D)** PAT extruder and **(E)** PAT conveyor line, after 7, 14, 21 and 28 days of compressed yeast production. Mean bacterial counts followed by the same superscripts are not significantly different ($P > 0.05$).

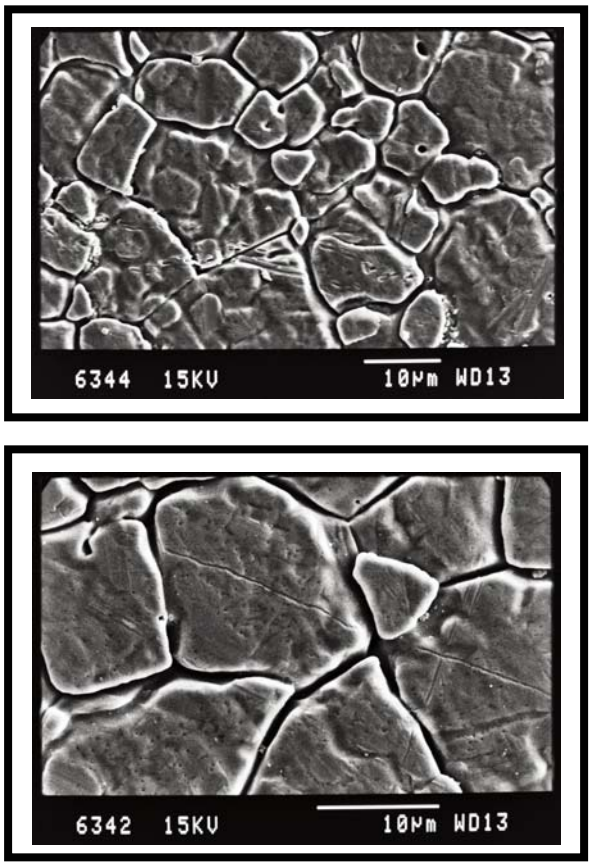


Figure 4.7 Scanning electron micrographs of an uncolonised stainless steel surface (control)

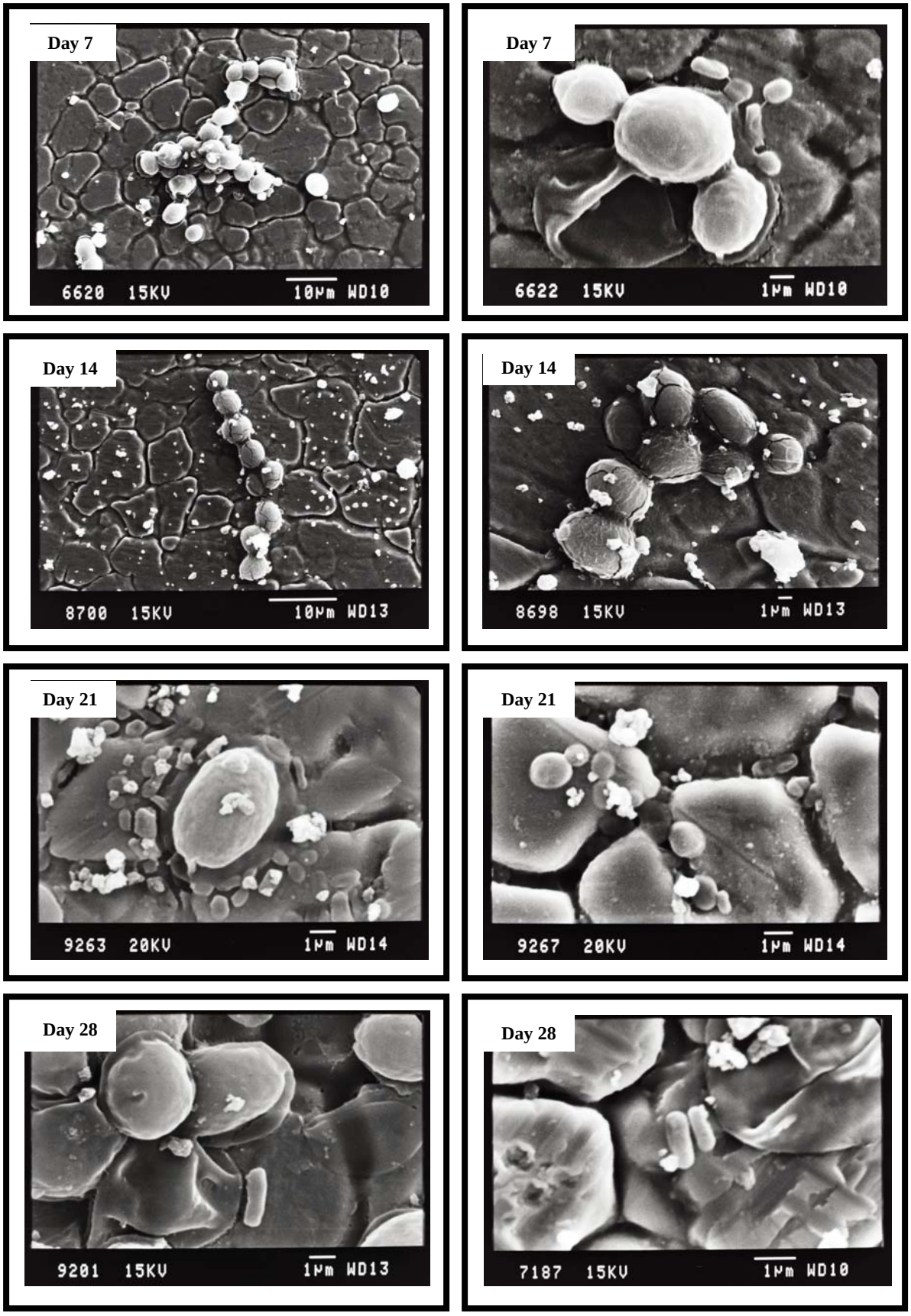


Figure 4.8 SEM of stainless steel surfaces sampled from the filter hopper **(1A)** after 7, 14, 21 and 28 days of Baker's compressed yeast production.

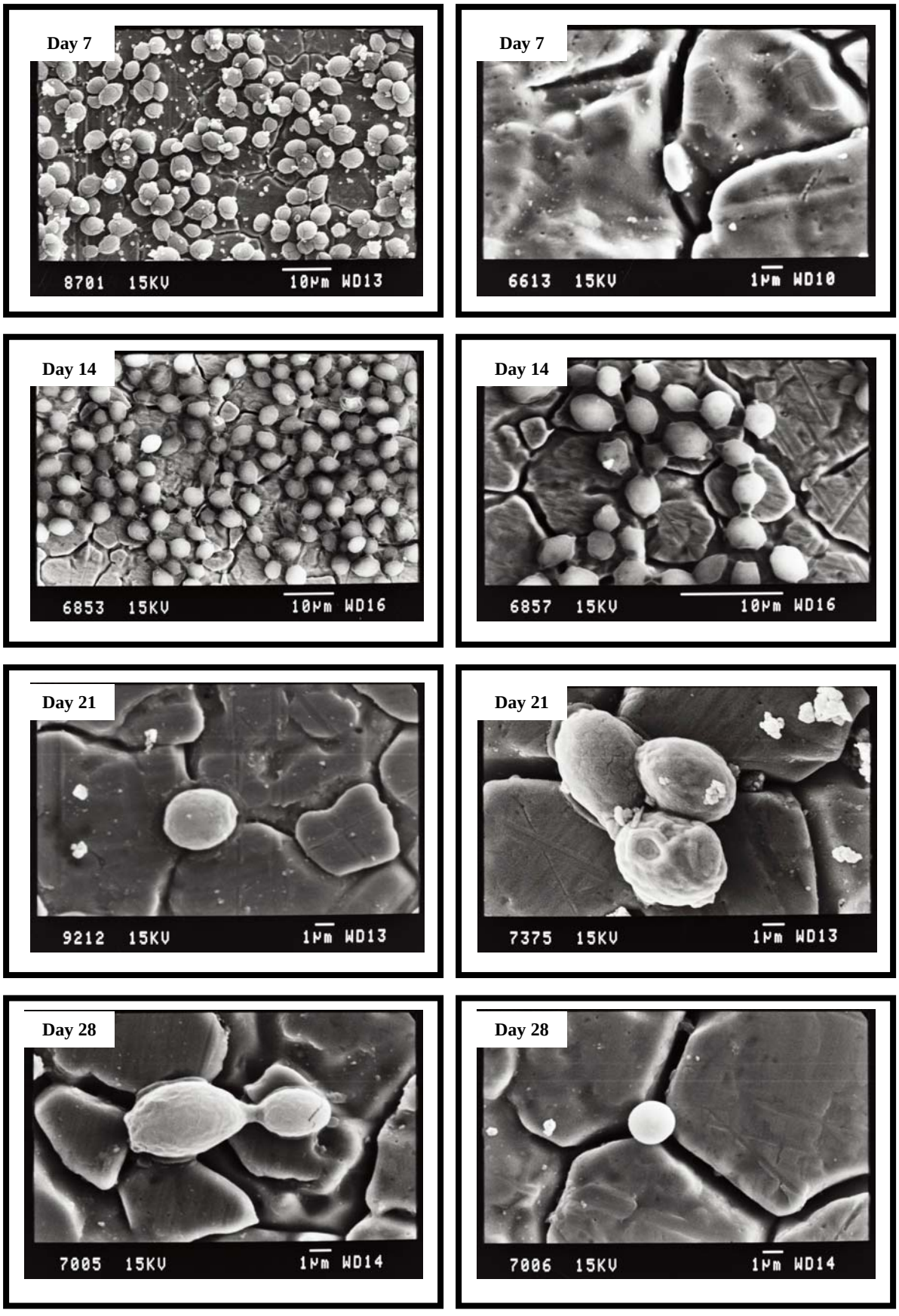


Figure 4.9 SEM of stainless steel surfaces sampled from the filter conveyor line **(1B)** after 7, 14, 21 and 28 days of Baker's compressed yeast production.

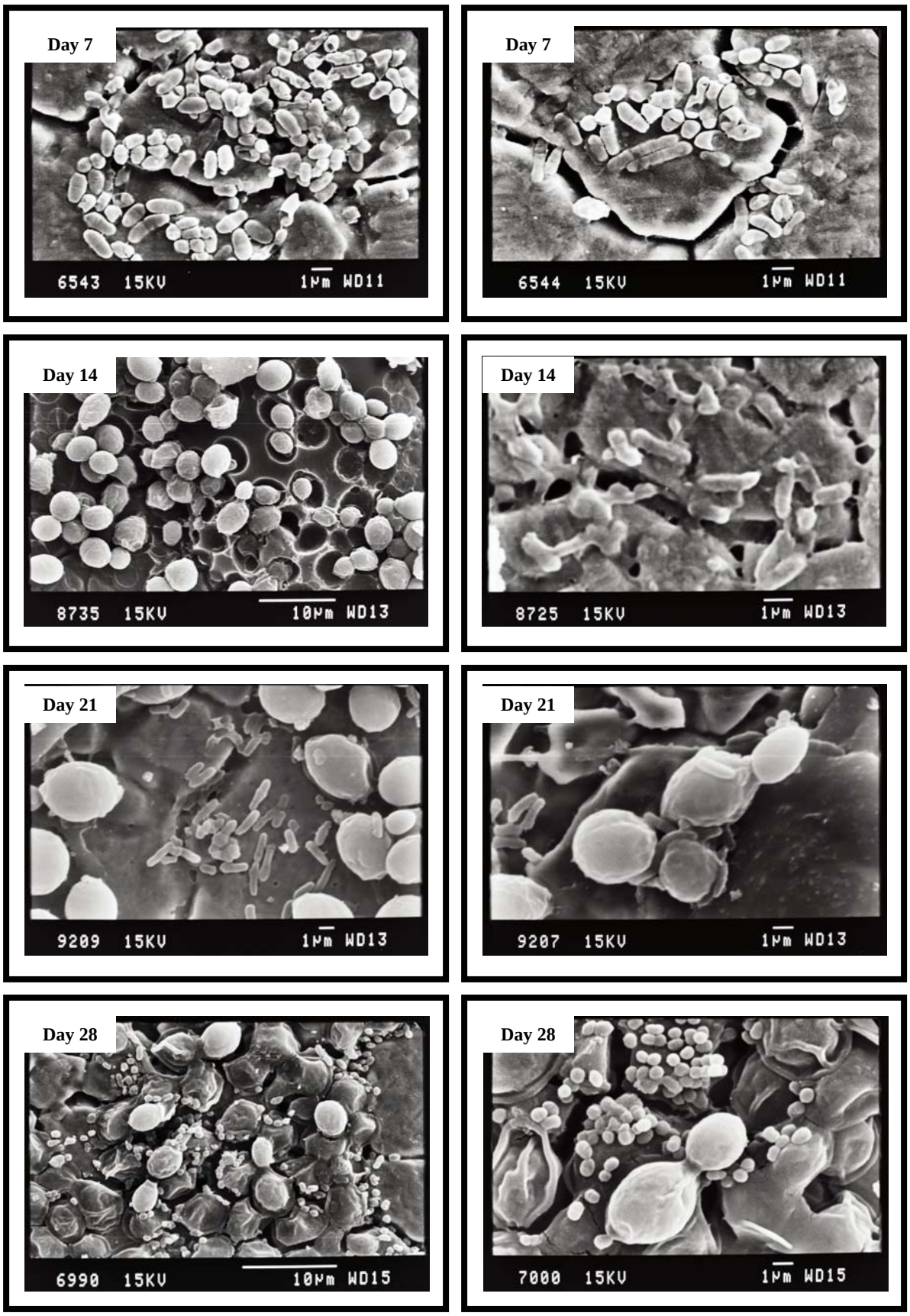


Figure 4.10 SEM of stainless steel surfaces sampled from the PAT hopper **(1C)** after 7, 14, 21 and 28 days of Baker's compressed yeast production.

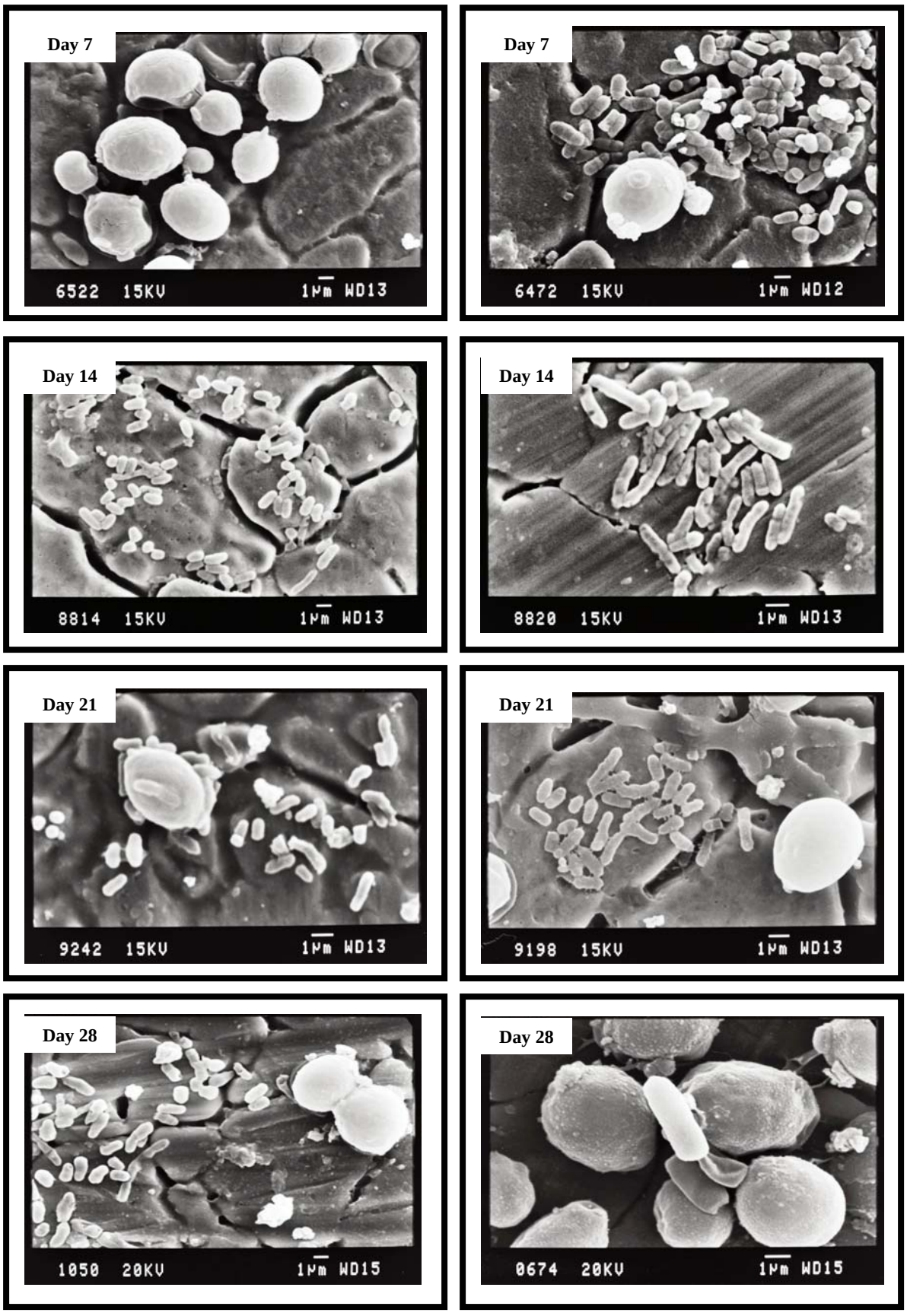


Figure 4.11 SEM of stainless steel surfaces sampled from the PAT extruder **(1D)** after 7, 14, 21 and 28 days of Baker's compressed yeast production.

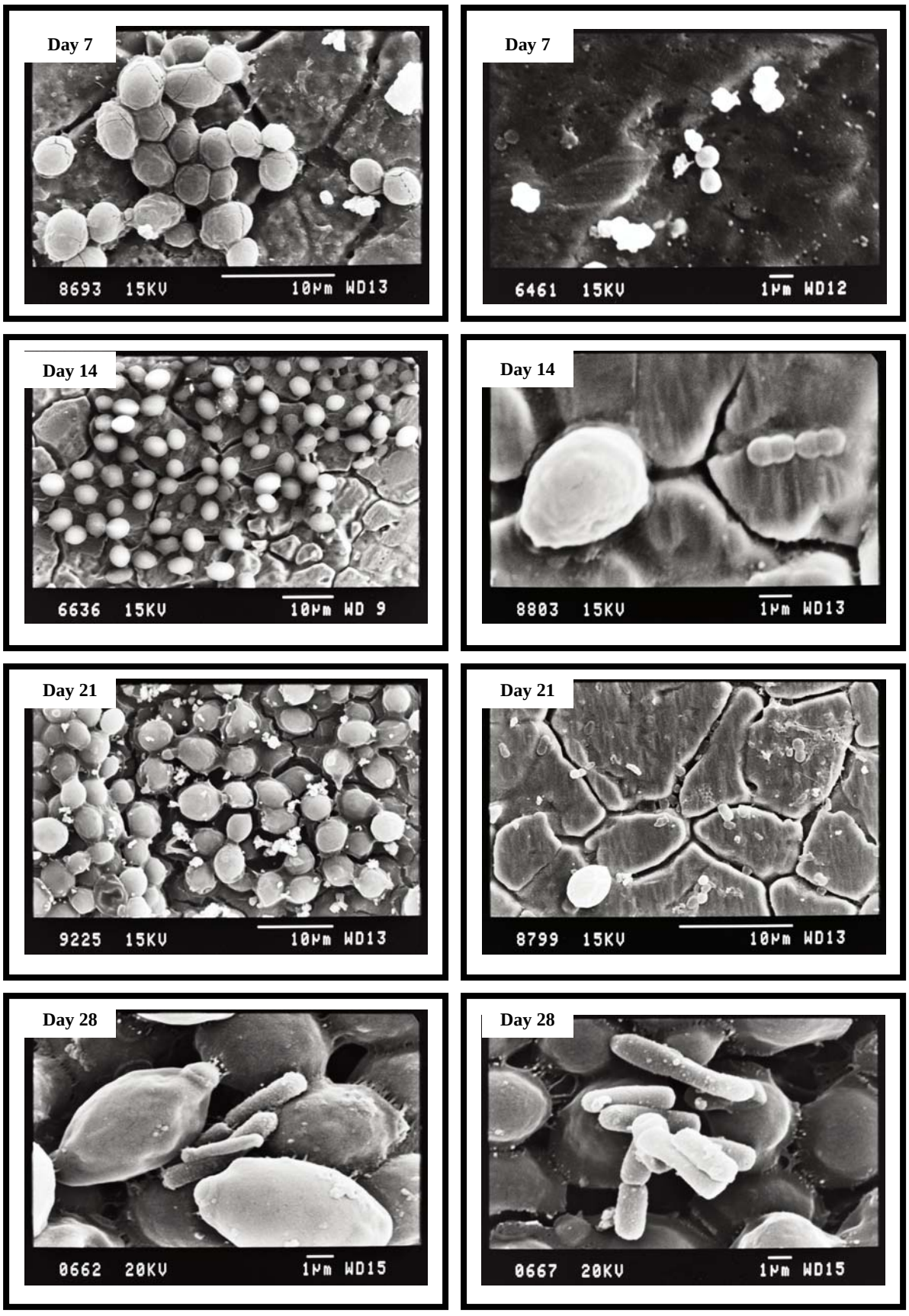


Figure 4.12 SEM of stainless steel surfaces sampled from the PAT conveyor line **(1E)** after 7, 14, 21 and 28 days of Baker's compressed yeast production.

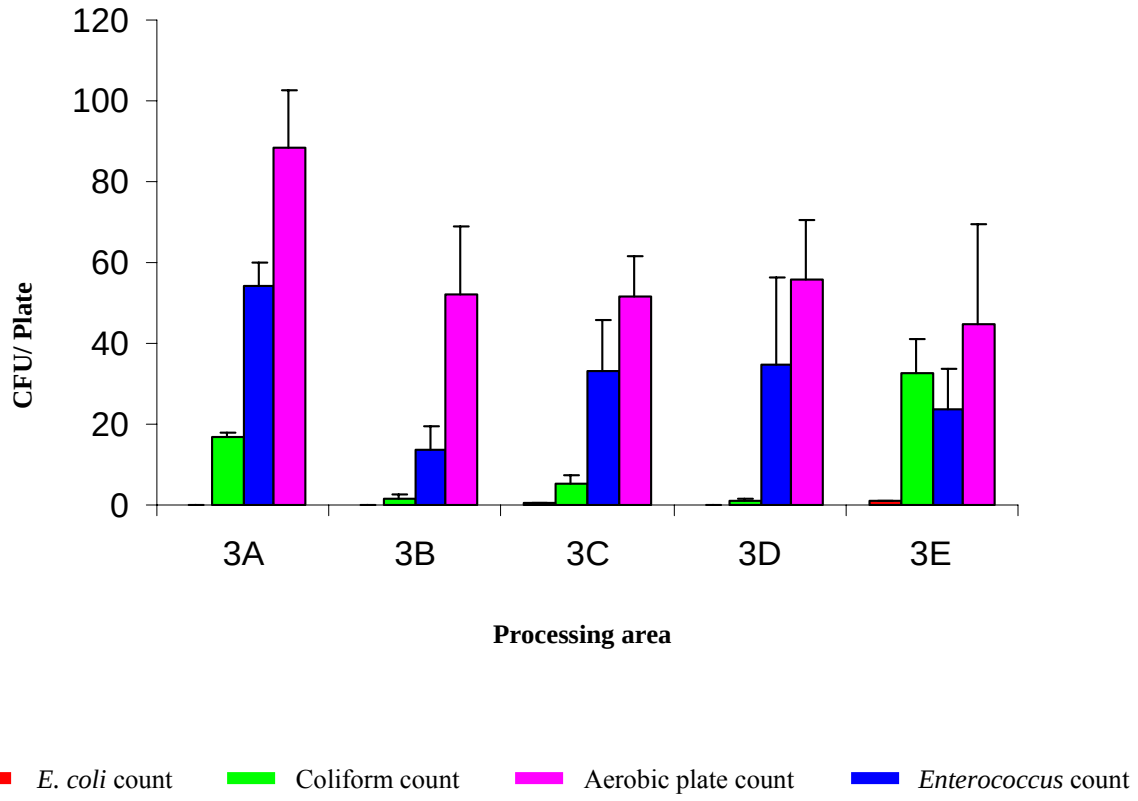


Figure 4.13 Mean numbers of *E. coli*, coliforms, *Enterococcus* and aerobic bacteria in the air adjacent to the **(3A)** Filter conveyor line, **(3B)** Overhead filter conveyor line/ hopper, **(3C)** PAT extruder, **(3D)** PAT conveyor line (start) and **(3E)** PAT conveyor line (end).

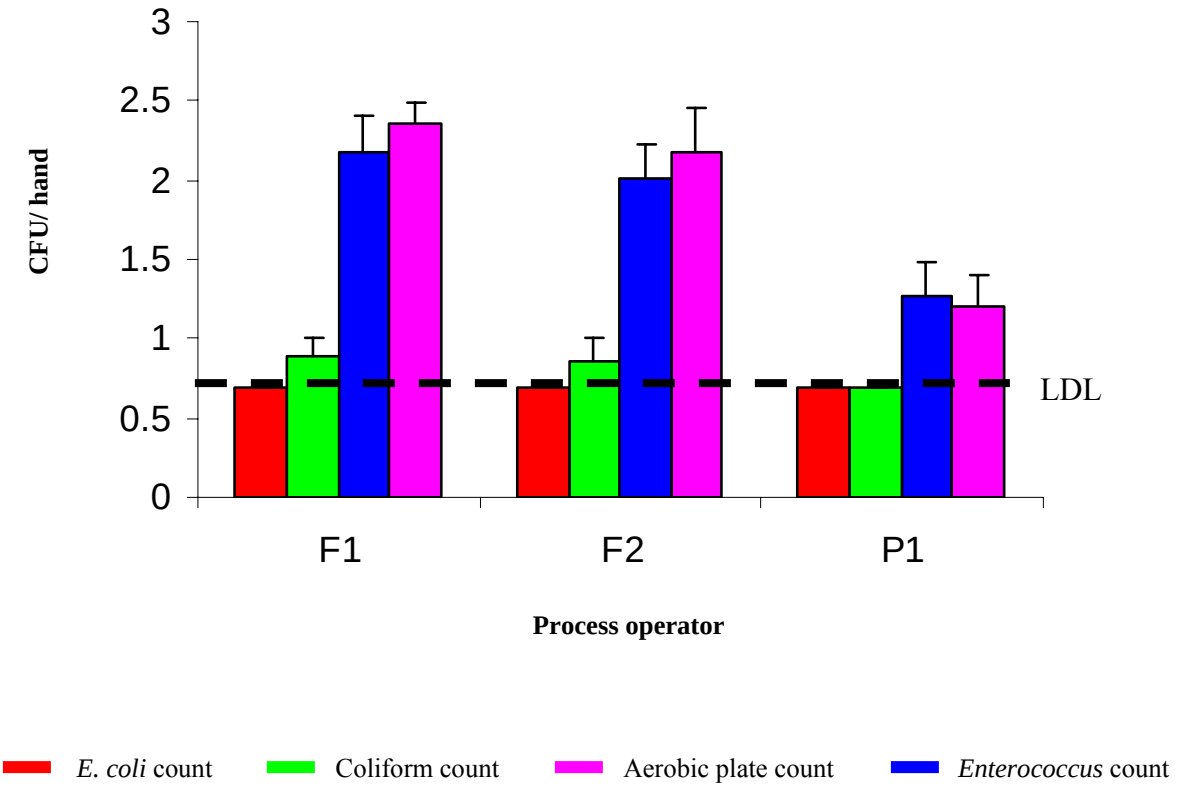


Figure 4.14 Mean numbers of *E. coli*, coliforms, *Enterococcus* and aerobic bacteria on workers' hands: **(F1)** Filter operator 1, **(F2)** Filter operator 2 and **(P1)** PAT operator 1. (Lower detection limit (LDL) = 0.7 log CFU/ hand – –)

Table 4.1 Percentage distribution of 318 isolates from aerobic plate counts of Baker's compressed yeast equipment surfaces using the 'mock' surface (180 isolates) **(A)** and swab (138 isolates) **(B)** technique

A

Day	1A			1B			1C			1D			1E			Total		
	%Ent[a]	*%Lac*[b]	%GN[c]	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN
7	63	37	0	17	83	0	17	0	83	8	50	42	50	50	0	**31**	**44**	**25**
14	50	50	0	0	0	0	75	25	0	100	0	0	75	25	0	**75**	**25**	**0**
21	100	0	0	100	0	0	100	0	0	75	25	0	50	50	0	**85**	**15**	**0**
28	100	0	0	100	0	0	84	8	8	33	0	67	100	0	0	**83**	**2**	**15**
Total	**78**	**22**	**0**	**72**	**28**	**0**	**69**	**8**	**23**	**54**	**19**	**27**	**69**	**31**	**0**	**68**	**22**	**10**
%Gram-positive		**100**			**100**			**77**			**73**			**100**			**90**	

B

Day	2A			2B			2C			2D			2E			Total		
	%Ent[a]	*%Lac*[b]	%GN[c]	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN	*%Ent*	*%Lac*	%GN
7	50	25	25	0	100	0	100	0	0	75	25	0	75	25	0	**60**	**35**	**5**
14	75	17	8	92	8	0	92	8	0	0	0	100	100	0	0	**72**	**7**	**21**
21	63	37	0	100	0	0	25	25	50	50	50	0	75	25	0	**63**	**27**	**10**
28	92	8	0	100	0	0	76	13	13	50	50	0	87	13	0	**81**	**17**	**2**
Total	**70**	**22**	**8**	**73**	**27**	**0**	**73**	**11**	**16**	**44**	**31**	**25**	**84**	**16**	**0**	**69**	**21**	**10**
%Gram-positive		**92**			**100**			**84**			**75**			**100**			**90**	

[a] *Enterococcacea* [b] *Lactobacillus* [c] Gram-negative rod

CHAPTER FIVE

SUMMARIZING DISCUSSION AND CONCLUSIONS

The production of commercial yeast for baking, wine and brewing fermentations is an extensive international industry. The final product is not always a pure culture, and can be contaminated with various levels of bacteria, including lactic acid bacteria (LAB) and coliform organisms. Although these quality issues are well recognized in the industry, there is little published information on the topic, especially on the source(s) and growth of these contaminants during the process of yeast manufacture.

Raw materials, processing equipment surfaces, air and workers' hands as sources of bacterial contamination in commercial yeast manufacturing

Part of this study aimed at tracking potential sources of bacterial contamination of commercially manufactured cream, compressed and dry yeast product. In achieving this objective, the ability to detect low levels of contaminants was imperative. Gram-negative indicator organisms, such as *E. coli* and other coliforms, contaminated yeast product at very low levels often being non-detectable on the day of sampling. In addition, standard plating procedures were generally not sensitive enough to detect low-level contamination in line-samples or finished product. This study made use of a preliminary incubation technique (O'Brien *et al.*, 2004a), based on the Virginia Shelf-life test for milk analysis, to boost bacterial counts associated with raw materials and production-line samples. Overall, significant increases ($P < 0.05$) in counts were observed for samples pre-incubated at 37°C for 24h, which assisted in the detection of bacterial contaminants and in finding the potential sources and key processing sites of contamination.

The quality of commercially manufactured yeast is dependent upon the quality of the raw materials used. In our study, raw materials used in the fermentation stages of yeast production, for example the yeast inoculum, process air, vitamins, minerals and process water, were negligible in contributing to the bacterial contamination of the yeast product. The raw molasses, a by-product obtained from the sugar industry, and directly transferred to an external holding tank, contained 2 log CFU/ ml enterococci and aerobic bacteria, along with spore-forming *Bacillus* spp. (results not shown). The high level of contamination observed for raw molasses showed the importance of a pre-sterilization step (± 127°C for 30 min) before entry into seed and commercial fermentations as the primary source of nutrients for dividing yeast cells. Furthermore, the current

holding temperature (*ca.* 65°C) used to store molasses (post-sterilization) appeared to be efficient in preventing any recontamination.

The soda ash, used to control the pH of larger seed and commercial fermentations was thought to be an unlikely source of contamination, due to its extreme alkalinity (*ca.* pH 11) and high holding temperature (*ca*. 60°C). However, on all three sampling occasions the soda ash was found to contain 2 log CFU/ ml enterococci. It is questionable whether the enterococci initially detected in seed yeast fermentations originated from the soda ash used to control pH levels. Since the pH of early seed yeast fermentations can be as low as 4 to 5, it is uncertain whether these alkaline tolerant isolates would survive under such different pH conditions. Future studies of these extreme alkolophilic and temperature tolerant enterococci isolates is required, which would include the use of molecular methods, such as DNA-sequencing and specific hybridization with 16S rDNA probes (Manero and Blanch, 2002), to establish whether the strains isolated from the soda ash in fact match those isolated from the seed yeast fermentations.

Using the preliminary incubation technique, the Mother fermentation (MF) was found to be the initial site of seed yeast contamination with *Enterococcus* and aerobic bacteria (*ca.* 2 log CFU/ ml). At this stage of yeast processing the initial level of contamination is most important, since the growth conditions used to grow up yeast biomass (pH 4 - 6, 25 - 35°C), can also be favourable to a wide range of acid tolerant bacteria (Tessendorf, 1991; Enfors, 2001). If levels of bacterial contaminants are high enough they could compete with the yeast cells for nutrients and result in extremely high numbers, which would not be as easily eradicated by ordinary acid treatment. In most food processes, manufacturers make use of hurdle technology in an attempt to remove contaminants from end-products by means of temperature treatment (e.g. cooking, pasteurization or freezing), chemical or physical preservation. However, in commercial yeast manufacturing, the end-product is a live culture of yeast cells, which eliminates the use of most processing and preservation regimes. In other words, any bacterial contaminants established in the product during manufacturing generally reside within the finished yeast product. In this study, current cleaning regimes for seed yeast fermentation vessels include rinsing with hot water and steam sterilization under pressure (*ca.* 80 kPa) without clean-in-place (CIP) equipment or use of detergents or sanitizers. It is therefore suggested that future methods of cleaning seed yeast

vessels, include the use of a mild alkaline detergent preferably with CIP equipment. This would assist in the removal of yeast product debris on the inner walls of the vessel which could otherwise protect bacterial contaminants, such as enterococci, from standard cleaning regimes.

The observed steadiness of *Enterococcus* and aerobic bacterial counts following commercial fermentation, was thought to be due to the reduced temperature (*ca.* 5°C) used during further processing of the cold-stored yeast cream into compressed and dry yeast product, which largely inhibited the proliferation of existing bacterial contaminants. Several processing sites were, however, highlighted as areas of increased product contamination or areas which supported the proliferation of existing bacterial contaminants. In most cases, increased product counts correlated with increased complexity of difficult-to-clean production equipment along the processing line. For example, in cream and compressed yeast production, the cold-storage of yeast cream after separation was the initial site during processing where contamination with Gram-negative coliforms organisms including *E. coli* was observed. The cold-storage tank in this study was similar in structure to those previously documented as difficult to clean and potential sources of bacterial contamination (Yoshida *et al*., 1998; Franz *et al.*, 2003). It has therefore been suggested, based upon the results obtained in this study, that the cold-storage tanks be cleaned more efficiently and between batches in order to eliminate the possibility of yeast product build-up.

During final stages of production, the hopper used to mix the yeast cake with emulsifier before extrusion as 5 kg blocks of Baker's compressed yeast, was a significant ($P < 0.05$) source of increased product contamination with coliforms. For wet product, poorly designed hoppers can create product hold-ups, which if allowed to remain, can become a significant source of contamination (Troller, 1983). In addition, the inaccessibility of this machine to proper cleaning can contribute to the possibility that it may harbor a variety of bacterial contaminants. This finding was confirmed by scanning electron microscopy (SEM) of the stainless steel surfaces associated with the hopper and extruder used in the commercial manufacturing of Baker's compressed yeast. Bacteria associated with these yeast processing equipment surfaces will be discussed in more detail. Similarly, in the final stages of dry yeast production coliform counts increased significantly ($P < 0.05$) after the storage and packaging of dried yeast. This finding was

in agreement with a previous study of the same commercial yeast factory (O'Brien *et al*., 2004a), and is not the first report of finished product contamination with Gram-negative bacteria, for example Enterobacteriaceae in vacuum-packaged vienna sausages (Dykes *et al.*, 1991) and *Serratia* spp. in poultry (Georgnaras *et al.*, 1996).

Overall, the analysis of the processing environment surrounding the final stages of Baker's compressed yeast production, confirmed the potential of air adjacent to the processing equipment as a potential source for low-level airborne contamination with *Enterococcus*, coliforms and *E. coli*. It has therefore been suggested that these processing areas be enclosed, in an attempt to reduce the flow of traffic (i.e. personal and fork-lifts) and subsequent air flow over the passing yeast product. This could otherwise result in the airborne contamination and surface spoilage of Baker's compressed yeast. The contribution of workers' hands, if brought into contact with passing yeast product, were found to be insignificant ($P > 0.05$) sources of compressed yeast product contamination. However, it is still recommended that workers within the processing area be trained in accordance with good manufacturing practices (GMP) and personal hygiene.

Processing equipment surfaces associated with the final stages of Baker's compressed yeast production were evaluated for the formation of yeast and bacterial biofilms. The use of stainless steel 'mock' surfaces established the presence and degree of biofilm formation on yeast processing equipment surfaces. Bacterial counts were highest after 28 days of exposure, exemplifying the accumulation of bacterial cells on yeast processing equipment surfaces over time. This was also true for yeast cells, whose attachment to the stainless steel as documented by SEM increased with time. However, counts indicated that the maximum level of attachment of *S. cerevisiae* to the stainless steel was 5 log CFU/ cm^2. This study confirmed the accumulation and attachment of yeast and bacterial cells to processing equipment surfaces, identifying the inefficiency of current methods of cleaning the processing equipment associated with Baker's compressed yeast production. However, this study found little evidence of (i) extracellular polymeric substance (EPS) production by yeast or bacterial cells, characteristic of mature biofilm formation and (ii) the formation of a binary species biofilms between yeast and bacterial cells. In fact, SEM images established that in most instances single bacterial cells were entrapped by the

attached yeast cells and within the crevices of stainless steel, and later forming small microcolonies.

Our study found SEM to be a useful tool to assess the accumulation of yeast product on equipment surfaces and highlighted the need for more efficient cleaning processes, especially in inaccessible areas, such as the hopper and extruder, which could otherwise harbor biofilms of spoilage or potentially pathogenic bacteria (O'Brien *et al.*, 2004b). Together with the use of 'mock' surfaces, SEM could be used as a qualitative tool for the assessment of cleanliness or biofilm formation on processing equipment surfaces. This study focused on the biofilms associated with Baker's compressed yeast production alone. However, the attachment of bacterial cells to equipment surfaces found downstream of the compressed yeast production line, suggests the probability exists that biofilms may form on the inner walls of fermentation vessels and storage tanks, processing areas highlighted as potential sources of bacterial contamination in this study. For future work it would be interesting to compare these results with biofilms formed on the equipment surfaces associated with the final stages of Brewer's dry yeast production.

A comparison of counts obtained using the 'mock' surface technique and swabbing technique, established (as is well documented) that swabbing was the less accurate method in enumerating total attached populations (Moore and Griffith, 2002). However, for ease of use, it is suggested that swabbing of equipment surfaces after cleaning and prior to yeast processing be incorporated by the yeast factory, in an attempt of monitoring the cleanliness of processing equipment surfaces.

In addition to the obvious potential of introducing spoilage and potentially pathogenic organisms into passing product, bacteria attached to processing equipment surfaces are also notorious for being agents of biofouling and surface corrosion (Kumar and Anand, 1998; Assanta *et al.*, 2002). This could result in shorter life-spans of processing equipment and financial loss with the necessary repair or replacement of processing equipment. In avoiding both the quality and financial aspects attached to the formation of persistent biofilms, it is essential for yeast manufacturers to assess the current methods of cleaning processing equipment surfaces. Since biofilms may consist of a mixed population of Gram-positive and Gram-negative organisms, an

investigation of the nature of a biofilm could facilitate the choice of an effective sanitizer (Gibson *et al*., 1999). Results obtained in this study indicated that levels of Gram-negative bacteria present in the product and around the processing area were low. It is therefore suggested that yeast manufacturers implement detergents and sanitizers specifically aimed at Gram-positive bacteria into their cleaning regime. However, the application of a sanitizer can be ineffective if there is a high concentration of 'soil' on the surface. It is therefore imperative that the yeast product residue be completely removed prior to sanitation. Consistent and efficient mechanical cleaning, followed by a sanitizer, would prevent product build-up and eliminate processing equipment surfaces as additional sources of contamination.

Shelf-life of commercially manufactured yeast products

In addition to producing a high quality yeast for baking and brewing, it is an important objective for yeast manufacturers to increase product shelf-life. Results from our study showed that storage temperature and time considerably influenced bacterial growth patterns associated with commercially manufactured fresh yeast. Bacterial spoilage and sensory deterioration was observed during lengthened storage periods, and especially at higher storage temperatures. Even when stored at 10°C, cream and compressed yeast samples became bacteriologically and visually spoiled. It was therefore apparent that any break in the cold-chain during processing, storage or distribution could affect the development of spoilage bacteria resulting in shortened product shelf-life of commercially manufactured fresh yeast. Conversely, this study found vacuum-packaged dry yeast to be the most bacteriologically stable product manufactured.

The bacteriological shelf-life of commercially manufactured yeast, until the present study, has not been fully quantified. In general, local limitations and recommended guidelines for commercially manufactured yeast products are not consistent, either with international specifications or with the shelf-life and usability of the finished product in baking and brewing. Since aerobic counts, even when yeast was visibly spoilt, complied with recommended guidelines (8 – 9 log CFU/ ml or g), we suggest limits for aerobic counts be decreased, in accordance with the numbers obtained in this study which were found to quantitatively represent the initiation of spoilage of cream and compressed yeast products (*ca.* 6 log CFU/ ml or g).

Predominant bacterial populations associated with the manufacturing of commercial yeast

Bacteria isolated from seed, cream, compressed and dry yeast samples, as well as those associated with the processing equipment surfaces in this study were predominantly Gram-positive members of the LAB family (81 – 100%), specifically *Lactobacillus* and *Enterococcaceae* (includes members of *Enterococcus*, *Lactococcus, Leuconostoc* and *Pediococcus*). It was clear that populations of *Lactobacillus* predominated during the early stages of yeast production, whilst proportions of *Enterococcaceae* increased during processing stages where excess water was removed from the yeast product (e.g. separation, filtering and drying). It is therefore apparent that during processing, as the a_w of the yeast product decreases the proportions of *Enterococcaceae* increase. The importance of this finding is not clear from the scope of this study, however it does suggest that the less desirable bacterial contaminants (i.e. members of the *Enterococcaceae*) outnumbered the more desirable populations of *Lactobacillus* during the final stages of production, having consequences in the use of yeast as a raw material in wine and brewing fermentations. It was a noteworthy finding that the bacterial populations isolated from the processing equipment surfaces were the same as those present in Baker's compressed yeast. It can be speculated that the bacteria present on the processing equipment surfaces investigated in this study, originated from the passing yeast product, possibly becoming a persistent source of contamination between batches.

From the shelf-life investigations it was clear that cream and compressed yeast were spoiled by LAB, which grew to high numbers during refrigerated storage, resulting in premature spoilage and costly losses to the manufacturer. The growth of LAB is favoured in cream and compressed yeast products because of their tolerance of low pH values (Reed and Nagodawithana, 1991), cold-storage temperatures (Jay, 1998) and in dry yeast products their ability to survive under microaerophilic conditions (Varnam, 2002). Results from this study showed LAB, predominantly *Lactobacillus*, to be responsible for decreasing the shelf-life of commercially manufactured fresh cream and compressed yeast products. *Micrococcus*, *Bacillus* and Gram-negative rods were found in low proportions in all three yeast products, and are therefore not suggested as primary organisms responsible for the spoilage of commercially manufactured yeast. Our results found that storage temperature, a_w and Eh affected the bacterial populations associated with the storage of commercially manufactured yeast. Where *Lactobacillus* dominated in yeast products with

higher storage temperature, a_w and Eh, whilst *Enterococcaceae* predominated under lower storage temperatures, a_w and Eh. Since final spoilage populations of commercially manufactured yeast were dominated by homofermentative lactobacilli and *Enterococcaceae*, it is suggested that control measures to reduce bacterial spoilage be aimed specifically at these Gram-positive genera. Since members of the LAB family, for example lactobacilli, grow well in dough and can be a cause of spoilage before baking (Varnam, 2002).

Foodborne pathogens associated with commercially manufactured yeast products

To assess the safety of commercially manufactured yeast intended for baking and brewing purposes, the status of foodborne pathogens was determined for each of the three commercial yeast products. In an attempt to detect the presence of these pathogens, the use of a preliminary incubation step was incorporated into the study. Results from this study found that preliminary incubation of samples before analysis for bacterial pathogens, increased the recovery of foodborne pathogens from yeast products. The cream yeast product, possibly due to the closed nature of its production in comparison to compressed and dry yeast, was found to be free of bacterial pathogens – making cream yeast the 'safest' of the three commercial yeast products. To our knowledge, this is the first report of *Listeria* (*L.*). *monocytogenes* isolation from both Baker's compressed and Brewer's dry yeast products. The risk of using yeast containing *L. monocytogenes* in baking appears to be small, since temperatures are generally high enough to kill most vegetative cells (Bailey and von Holy, 1993). However, the use of this yeast in wine and brewing fermentations may have food safety implications. Spore-forming *Bacillus* (*B.*) *subtilis*, *B. licheniformis* and *B. megaterium*, responsible for the rope-spoilage of baked products, were isolated from vacuum-packaged dry yeast in this study. Before control measures for bacterial pathogens are investigated or even instituted, efforts should be focused on prevention of contamination of Baker's and Brewer's yeast with pathogenic bacteria in the yeast processing environment. Based on similar studies of food processing environments, it is speculated that contamination of yeast products with *L. monocytogenes*, may originate from the processing environment (Lundén, 2004), whilst contamination with *Bacillus* spores may be from the atmosphere (Collins *et al*., 1991; Bailey and von Holy, 1993).

Practical implications of this study

There is constant pressure on manufacturers to increase the local and international exportation of commercially manufactured yeast for baking and brewing purposes. We recommend that several processing areas be sites for alteration of cleaning regimes and increased inspection, in an attempt to reduce the current level of bacterial contamination associated with commercially manufactured yeast, which could otherwise compromise its entry into international markets. These processing areas include the fermentation vessels, cold-storage tanks, hoppers, extruders and packaging equipment. The soda-ash, used to control the pH of fermentations, was highlighted as a potential source of yeast product contamination with *Enterococcus* in this study. It is suggested that the source of this raw material be re-evaluated, together with the installation of a new holding tank, which could otherwise be a source of persistent contamination to future yeast fermentations. Shelf-life investigations highlighted the effect of increased temperature on the shelf-life of fresh yeast. We recommend that the manufacturer incorporate a temperature monitoring program, in an attempt to maintain refrigerated temperatures (< 5°C) throughout the production, storage and distribution of cream and compressed yeast products.

Future work

This work has well established the processing areas which are in need of increased attention. Future research should, however, focus on biofilms associated with other yeast manufacturing equipment, for example fermentation vessels, storage tanks and dry yeast processing surfaces. Furthermore, the efficacy of various sanitizers and/ or a combination thereof would help industry eradicate the microbial biofilms associated with yeast processing equipment surfaces. The source(s) of *Listeria monocytogenes* and *Bacillus* spores in Baker's compressed and Brewer's dry yeast was not investigated in this study. Future work should focus on identifying the origin of these bacterial pathogens in commercial yeast, which could have safety implications in its use in baking and brewing. Lastly, future investigations should include characterization studies on the alkaline tolerant enterococci isolated from the soda ash in this study.

Conclusions of this study

This work documents an extensive microbiological survey of a typical commercial yeast factory, which could also be used as a model approach for any food manufacturing process, for the

investigation of sources of product contamination, incorporating the use of preliminary incubation of samples for the detection of low levels of contaminants or pathogens. This investigation established some critical points in the commercial manufacturing of yeast. Results from this study provide information that may be useful in reducing the number of spoilage and potentially pathogenic bacteria associated with Baker's and Brewer's yeast. However, in an attempt to reduce the predominantly Gram-positive populations associated with commercial yeast products and the processing environment it is important to remember that these contaminants are more desirable than the type of bacteria that they have replaced. In achieving a yeast product of high quality and safety together with a longer shelf-life, complete integration and coordination between cleaning programs and manufacturing operations are critical not only to a successful but also profitable business.

REFERENCES

Andrade, N. J., Bridgeman, T. A. and Zottola, E. A., 1998. Bacteriocidal activity of sanitizers against *Enterococcus faecium* attached to stainless steel as determined by plate count and impedance methods. Journal of Food Protection 61, 833 – 838.

Anwar, H., Strap, J. L., Costerton, J. W., 1992. Eradicating of biofilm cells of *Staphylococcus aureus* with tobramycin and cephalexin. Canadian Journal of Microbiology 38, 618 – 625.

Arihara, K., Ogihara, S., Sakata, J., Itoh, M. and Kondo, Y., 1991. Antimicrobial activity of *Enterococcus faecalis* against *Listeria monocytogenes*. Letters in Applied Microbiology 13, 190 –192.

Assanta, M. A., Roy, D., Lemay, M. J. and Montpetit, D., 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. Journal of Food Protection 65, 1240 – 1247.

Bailey, C. P. and von Holy, A., 1993. *Bacillus* spore contamination associated with commercial bread manufacture. Food Microbiology 10, 287 – 294.

Bale, M. J., Bennett, P. M., Beringer, J. E. and Hinton, M., 1993. The survival of bacteria exposed to desiccation on surfaces associated with farm buildings. Journal of Applied Bacteriology 75, 519 – 528.

Barrette, J., Champagne, C. P. and Goulet, J., 1999. Development of bacterial contamination during production of yeast extracts. Applied and Environmental Microbiology 65, 3261 – 3263.

Bascomb, S. and Manafi, M., 1998. Use of enzyme tests in characterization and identification of aerobic and facultatively anaerobic gram-positive bacteria. Clinical Microbiology Reviews 11, 318 – 340.

Birollo, G. A., Reinheimer, J. A. and Vinderola, C. G., 2001. Enterococci vs. non-lactic acid microflora as hygiene indicators for sweetened yoghurt. Food Microbiology 18, 597 – 604.

Boyd, A. and Chakrabarty, A. M., 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. Journal of Industrial Microbiology 15, 162 – 168.

Carpentier, B. and Cerf, O., 1993. Biofilms and their consequences with particular reference to hygiene in the food industry. Journal of Applied Bacteriology 75, 499 – 511.

Champagne, C. P., Barrette, J. and Goulet, J., 1999. Interaction between pH, autolysis promoters and bacterial contamination on the production of yeast extracts. Food Research International 32, 575 – 583.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. and Ghannoum, M. A., 2001. Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. Journal of Bacteriology, 183 (18) 5385 – 5394.

Characklis, W. G. and Marshall, K. C., 1990. Biofilms. John Wiley, New York, USA.

Chumkhunthod, P., Schraft, H. and Griffiths, M. W., 1998. Rapid monitoring method to assess efficacy of sanitizers against *Pseudomonas putida* biofilms. Journal of Food Protection 61, 1043 – 1046.

Clayton, B., 1973. The complete book of breads. Simon and Schuster, New York, USA.

Collee, J. G., Fraser, A. G., Marmion, B. P. and Simmons, A. (Eds.), 1996. Mackie & McCartney Practical Medical Microbiology, 14th Ed., Churchill Livingstone, London, UK.

Collins, N. E., Kischner, L. M. and von Holy, A., 1991. Characterization of *Bacillus* isolates from ropey bread, bakery equipment and raw materials. South African Journal of Science 87, 62 – 66.

Costerton, J. W., 1995. Overview of microbial biofilms. Journal of Industrial Microbiology 15, 137 – 140.

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. and Marrie, T. J., 1987. Bacterial biofilms in nature and disease. Annual Review of Microbiology 41, 435 – 464.

Costerton, J. W., Lewandowski, Z., de Beer, D., Caldwell, D., Korber, D. and James, G., 1994. Biofilms, the customized microniche. Journal of Bacteriology 176, 2137 – 2142.

Daniel, A. R., 1972. Bakery questions answered. Applied Science Publishers Ltd., London, UK.

Devriese, L. A., Collins, M. D. and Wirth, R., 1991. The genus *Enterococcus*. In: Balows, A., Trüper, H. G., Dworkin, M., Harder, W., Schleifer, K. H. (Eds.), The Prokaryotes. Springer-Verlag, New York, pp. 1465 – 1481.

Devriese, L. A., Pot, B., Van Damme, L., Kersters, K. and Haesebrouck, F., 1995. Identification of *Enterococcus* species isolated from foods of animal origin. International Journal of Food Microbiology 26, 187 – 197.

Doyle, M.P., Beuchat, L.R. and Montville, T. J. (Eds.), 1997. Food microbiology: fundamentals and frontiers, American Society for Microbiology, Washington, D.C.

Dunsmore, D. G., 1981. Bacteriological control of food equipment surfaces by cleaning systems. I. Detergent effects. Journal of Food Protection 44, 15 – 20.

Duyvesteyn,W. S., Shimoni, E. and Labuza, T. P., 2001. Determination of the end of shelf-life for milk using the Weibull hazard method. Lebensmittel-Wissenschaft und –Technologie 34, 143 – 148.

Dykes, G. A., Cloete, T. E. and von Holy, A., 1991. Quantification of microbial populations associated with the manufacture of vacuum-packaged, smoked vienna sausages. International Journal of Food Microbiology 13, 239 – 248.

Dykes, G. A., Cloete, T. E. and von Holy, A., 1993. Morphological characterization of lactic acid bacteria from spoiled, vacuum-packaged vienna sausages. Suid-Afrikaanse Tydskrif vir Wetenskap 89, 269 – 275.

Dykes, G. A., Marshall, L. A., Meissner, D. and von Holy, A., 1996. Acid treatment and pasteurization affect the shelf-life and spoilage ecology of vacuum-packaged vienna sausages. Food Microbiology 13, 69 – 74.

Enfors, S. –O., 2001. Baker's yeast. In: Ratledge, C. and Kristiansen, B. (Eds.), Basic Biotechnology, 2nd Ed., Cambridge University Press, UK, pp. 377 – 389.

Faber, J. M., 1991. Microbial aspects of modified-atmosphere packaging technology: a review. Journal of Food Protection 54, 58 – 70.

Fischer, P. L., Jooste, P. J. and Novello, J. C., 1986. Evaluation of rapid plate counting techniques for the enumeration of psychrotrophic bacteria in raw milk. South African Journal of Dairy Science 18, 137 – 141.

Flahaut, S., Hartke, A., Giard, J. –C. and Auffray, Y., 1997. Alkaline stress response in *Enterococcus faecalis*: adaptation, cross-protection, and changes in protein synthesis. Applied and Environmental Microbiology 63 (2), 812 – 814.

Frank, J. F. and Chmielewski, R. A. N., 1997. Effectiveness of sanitation with quaternary ammonium compound or chlorine on stainless steel and other domestic food-preparation surfaces. Journal of Food Protection 60, 43 – 47.

Franz, C. M. A. P., 1993. Heat resistance and inactivation of meat spoilage lactic acid bacteria. Master's Thesis, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.

Franz, C. M. A. P., Dykes, G. A. and von Holy, A., 1991. Effect of *in vitro* pH and temperature changes on meat spoilage lactic acid bacteria. South African Journal of Food Science and Nutrition 3, 59 – 62.

Franz, C. M. A. P., Holzapfel, W. H. and Stiles, M. E., 1999. Enterococci at the crossroads of food safety? International Journal of Food Microbiology 47, 1 – 24.

Franz, C. M. A. P., Stiles, M. E., Schleifer, K. H. and Holzapfel, W. H., 2003. Enterococci in foods – a conundrum for food safety. International Journal of Food Microbiology 88, 105 – 122.

Franz, C. M. A. P. and von Holy, A., 1996. Bacterial populations associated with pasteurized vacuum-packed vienna sausages. Food Microbiology 13 (2), 165 – 174.

Gall, K., Scott, V. N., Collette, R., Jahncke, M., Hicks, D. and Wiedmann, M., 2004. Implementing targeted good manufacturing practices and sanitation procedures to minimize *Listeria* contamination of smoked seafood products. Food Protection Trends 24 (5), 302 – 315.

Gelsomino, R., Vancanneyt, M., Cogan, T. M., Condon, S. and Swings, J., 2002. Source of enterococci in a farmhouse raw-milk cheese. Applied and Environmental Microbiology 68, 3560 – 3565.

Geornaras, I., de Jesus, A. E., van Zyl, E. and von Holy, A., 1996. Bacterial populations associated with poultry processing in a South African abattoir. Food Microbiology 13, 457 – 465.

Geornaras, I., de Jesus, A. E. and von Holy, A., 1998. Bacterial populations associated with the dirty area of a South African poultry abattoir. Journal of Food Protection 61 (6), 700 – 703.

Gibson, H., Taylor, J. H., Hall, K. E. and Holah, J. T., 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. Journal of Applied Microbiology 87, 41 – 48.

Gilbert, P., Das, J. and Foley, I., 1997. Biofilm susceptibility to antimicrobials. Advances in Dental Research 11, 160 – 167.

Gill, C. D. and Molin, G., 1991. Modified atmosphere and vacuum packaging. In: Russel, N. J. and Gould, G. W. (Eds.), Food Preservatives. Blackie, London, UK, pp. 172 – 199.

Gill, C. O. and Tan, K. H., 1980. Effect of carbon dioxide on growth of meat spoilage bacteria. Applied and Environmental Microbiology 39, 317 – 319.

Giraffa, G., Carminati, D. and Neviani, E., 1997. Enterococci isolated from dairy products: a review of risks and potential technological use. Journal of Food Protection 60, 1432 – 1438.

Greenberg, A. E. and Hunt, D. A. (Eds.), 1985. Laboratory Procedures for the Examination of Seawater and Shellfish, 5th Ed., The American Public Health Association, Washington, DC.

Guynot, M. E., Marín, S., Sanchis, V. and Ramos, A. J., 2003. Modified atmosphere packaging for prevention of mold spoilage of bakery products with different pH and water activity levels. Journal of Food Protection 66 (10), 1864 – 1872.

Haggblade, S. and Holzapfel, W. H., 1989. Industrialization of Africa's indigenous beer brewing. In: Steinkraus, K. H. (Ed.), Industrialization of Indigenous fermented foods. Marcel/ Dekker, New York, pp. 191 – 283.

Hartman, P. A., Deibel, R. H. and Sieverding, L. M., 1992. Enterococci. In: Vanderzant, C. and Splittstoesser, D. F. (Eds.) Compendium of methods for the microbiological examination of foods, American Public Health Association, USA, pp. 523 – 531.

Hassan, A. N., Birt, D. M. and Frank, J. F., 2004. Behavior of *Listeria monocytogenes* in a *Pseudomonas putida* biofilm on a condensate-forming surface. Journal of Food Protection 67 (2), 322 – 327.

Hautera, P. and Lovgren, T., 1975. The fermentation activity of baker's yeast. Its variation during storage. Bakers Digest 49, 36 – 37.

Hobbs, B. C. (Ed.), 1974. Food poisoning and food hygiene, 3rd Ed., Edward Arnold, London, pp. 22 – 32 and 264 – 274.

Hoffman, C., Schweitzer, T. R. and Dalby, G., 1973. Control of rope in bread. Industrial and Engineering Chemistry 29, 464 – 467.

Hood, S. K. and Zottola, E. A., 1997. Adherence to stainless steel by foodborne microorganisms during growth in model food systems. International Journal of Food Microbiology 37, 145 – 153.

Hough, J. S., 1985. The biotechnology of malting and brewing. Cambridge University Press, Cambridge, New York, pp. 93 – 111, 114 – 134.

Hough, J. S., Briggs, D. E., Stevens, R. and Young, T. W., 1982. Malting and brewing science, 2nd Ed., Chapman and Hall, New York, pp. 741 – 773.

Ingham, S. C. and Lau, M. M., 2003. Comparative survival of *Staphylococcus epidermidis*, *Listeria monocytogenes*, and *Staphyloccus aureus* on hot-smoked fish. Food Protection Trends 23 (9), 735 – 742.

Irvin, R., 1954. Commercial yeast manufacture. In: Underkofler, L. A., Hickey, R. J. (Eds.) Industrial Fermentations, Chemical Publishing Inc., New York, pp. 273 – 306.

James, G. A., Beaudette, L., Costerton, J. W., 1995. Interspecies bacterial interactions in biofilms. Journal of Industrial Microbiology 15, 257 – 262.

Janning, B. and Veld, P.H., 2001. Susceptibility of bacterial strains to desiccation: a simple method to test their stability in microbiological reference materials. Analytica Chimica Acta 286 (3), 469 – 476.

Jay, J. M. (Ed.), 1998. Modern Food Microbiology, 5th Ed., Van Nostrand Reinhold, New York.

Keller, S. E., Merker, R. I., Taylor, K. T., Tan, H. L., Melvin, C. D., Chirtel, S. J. and Miller, A. J., 2002. Efficacy of sanitation and cleaning methods in a small apple cider mill. Journal of Food Protection 65 (6), 911 – 917.

Kirschner, L. M. and von Holy, A., 1989. Rope spoilage of bread. South African Journal of Science 85, 425 – 427.

Knudtson, L. M. and Hartman, P. A., 1992. Routine procedures for isolation and identification of enterococci and fecal streptococci. Applied and Environmental Microbiology 58 (9), 3027 – 3031.

Kotze, M. A., 2002. Bacterial populations associated with equipment surfaces in processed meat operations. Honour's Dissertation, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.

Kumar, C. G. and Anand, S. K., 1998. Significance of microbial biofilms in food industry: a review. International Journal of Food Microbiology 42, 9 – 27.

Kunene, N. F., Hastings, J. W. and von Holy, A., 1999. Bacterial populations associated with a sorghum-based fermented weaning cereal. International Journal of Food Microbiology 49, 75 – 83.

Legan, J. D. and Voysey, P. A., 1991. Yeast spoilage of bakery products and ingredients. Journal of Applied Bacteriology 70, 361 – 371.

Lengeler, J. W., Drews, G. and Schlegel, H. G. (Eds.), 1999. Biology of the prokaryotes, Blakwell Science, New York, USA.

Leroi, F., Joffraud, J. – J., Chevalier, F. and Cardinal, M., 1998. Study of the microbial ecology of cold-smoked salmon during storage at 8°C. International Journal of Food Microbiology 39, 111 – 121.

Lewis, S. J., Gilmour, A., Frazer, T. W. and McCall, R. D., 1987. Scanning electron microscopy of stainless steel inoculated with single bacterial cells. International Journal of Food Microbiology 4, 279 – 284.

Lindsay, D., Geornaras, I. and von Holy, A., 1996. Biofilms associated with poultry processing equipment. Microbios 86, 105 – 116.

Lonner, C. and Preve-Akesson, K., 1989. Effects of lactic acid bacteria on the properties of sour dough bread. Food Microbiology 6, 19 – 35.

Lues, J. F. R., 1992. The effect of abiotic and biotic factors on the microbiological composition of Baker's yeast. Master's Thesis, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.

Lundén, J., 2004. Persistent *Listeria monocytogenes* contamination in food processing plants. PhD Thesis, Department of Food and Environmental Hygiene, University of the Helsinki, Finland.

Mafu, A. A., Roy, D., Goulet, J. and Hagny, P., 1990. Attachment of *Listeria monocytogenes* to surfaces. Applied and Environmental Microbiology 57, 1969 – 1973.

Manero, A. and Blanch, A. R., 1999. Identification of *Enterococcus* spp. with a biochemical key. Applied and Environmental Microbiology 65, 4425 – 4430.

Manero, A. and Blanch, A. R., 2002. Identification of *Enterococcus* spp. based on specific hybridization with 16S rDNA probes. Journal of Microbiological Methods 50, 115 – 121.

Marshall, K. C., Stout, R., Mitchell, R., 1971. Mechanism of the initial events in the sorption of marine bacteria to surfaces. Journal of General Microbiology 68, 337 – 348.

Marshall, R. T. (Ed.), 1992. Standard methods for the examination of dairy products, 16th Ed., American Public Health Association. Washington, D. C.

Martinez-Anaya, M. A., Pitarch, B., Bayarri, P. and Benedito de Barber, C., 1990. Microflora of the sourdoughs of wheat flour bread. X. Interactions between yeasts and lactic acid bacteria in wheat doughs and their effects on bread quality. Cereal Chemistry 67 (1), 85 – 91.

McEldowney, S. and Fletcher, M., 1987. Adhesion of bacteria from mixed cell suspensions to solid surfaces. Archives of Microbiology 148, 57 – 62.

Membré, J. M., Kubaczka, M. and Chéné, C., 1999. Combined effects of pH and sugar on growth rate of *Zygosaccharomyces rouxii*, a bakery product spoilage yeast. Applied and Environmental Microbiology 65 (11), 4921 – 4925.

Moore, G. and Griffith, C., 2002. Factors influencing recovery of microorganisms from surfaces by use of traditional hygiene swabbing. Dairy, Food and Environmental Sanitation 22, 410 – 421.

Morrison, D., Woodford, N. and Cookson, B., 1997. Enterococci as emerging pathogens of humans. Journal of Applied Microbiology Symposium Supplement 83, 89S – 99S.

Mossel, D. A. A., Corry, J. E. L., Struijk, C. B. and Baird, R. M. (Eds.), 1995. Essentials of the microbiology of foods: a textbook for advanced studies. John Wiley and Sons, England, pp. 350 – 355.

Notermans, S., Dormans, J. A. M. A., Mead, G. C., 1991. Contribution of surface attachment to the establishment of microorganisms in food processing plants: a review. Biofouling 5, 21 – 36.

Novellie, L., 1963. Bantu beer-food or beverages? Food Industry of Southern Africa 12, 28.

O'Brien, S. S., Lindsay, D. and von Holy, A., 2004a. The presence of *Enterococcus*, coliforms and *E. coli* in a commercial yeast manufacturing process. International Journal of Food Microbiology 94, 23 – 31.

O'Brien, S. S., Lindsay, D., von Holy, A., 2004b. Biofilm formation on yeast processing equipment surfaces as studied by scanning electron microscopy. Proceedings of the Electron Microscopy Society of Southern Africa 34, 39.

Oura, E., Suomalainen, H., and Viskari., R., 1982. Breadmaking. In: Rose, A. H. (Ed.) Economic Microbiology Volume 7: Fermented Foods, Academic Press Inc., London, pp. 88 – 143.

Pattison, T. -L., 2000. Efficacy of selected natural antimicrobials as bread preservatives. Master's Thesis, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.

Pattison, T. -L., Geornaras, I. and von Holy, A., 1998. Microbial populations associated with commercially produced South African sorghum beer as determined by conventional and Petrifilm™ plating. International Journal of Food Microbiology 43, 115 – 122.

Pattison, T. -L. and von Holy, A., 2001. Effect of selected natural antimicrobials on baker's yeast activity. Letters in Applied Microbiology 33, 211 – 215.

Peppler, H. J., 1982. Yeast extracts. Economic Microbiology 7, 293 – 312.

Pereira, M. O., Morin, P., Vieira, M. J. and Melo, L. F., 2002. A versatile reactor for continuous monitoring of biofilm properties in laboratory and industrial conditions. Letters in Applied Microbiology 34, 22 – 26.

Prescott, L. M., Harley, J. P. and Klein, D. A. (Eds.), 1996. Microbiology, 3rd Ed., Wm. C. Brown Publishers, U. S. A., pp. 97 – 129, 441 - 449 and 504 - 852.

Reed, G. and Nagodawithana, T. W. (Eds.), 1991. Yeast technology, 2nd Ed., The AVI Publishing Company Inc., New York, pp. 89 – 151 and 261 – 369.

Reuter, G., 1992. Culture media for enterococci and group D-streptococci. International Journal of Food Microbiology 17, 101 – 111.

Seiler, D. A. L., 1975. Product spoilage and hygiene. In: Brown, J (Ed.), The master baker's book to breadmaking, Herts and Hastings Printing Company, National Association of Master Bakers, pp. 441 – 453.

Shapton, D. A. and Shapton, N. F. (Eds.), 1991. Principles and practices for the safe processing of foods. Butterworth-Heinemann Ltd., London, UK, pp. 222 – 440.

Sleigh, J. D. and Timbury, M. C., 1998. Notes on Medical Bacteriology, 5th Ed., Churchill Livingstone, Edinburgh, UK, pp. 59 – 71 and 137 – 139

Sneath, P.H., Moir, N. S. G., Sharpe, M. E. and Holt, J. G., 1981. The Gram-positive cocci. In: Shleifer, K. H (Ed.), Bergeys Manual of Systematic Bacteriology Volume 2, William and Wilkens, Baltimore, USA.

South African Bureau of Standards, 1995. Baker's compressed yeast, SABS 1642, South African Bureau of Standards, Pretoria, South Africa.

South African Bureau of Standards, 1995. Dried yeast for use in wet sorghum beer brewing, SABS 1643, South African Bureau of Standards, Pretoria, South Africa.

Stiles, M. E. and Holzapfel, W. H., 1997. Lactic acid bacteria of foods and their current taxonomy. International Journal of Food Microbiology 36, 1 – 29.

Streuli, H. and Staub, M., 1955. Uber die Erreger der Brotkrankheit. Lebensmittel-Wissenschaft und –Technologie 46, 312 – 339.

Sugihara, T. F., 1985. Microbiology of bread making. In: Wood, B. J. B. (Ed.) Microbiology of fermented foods, Elsevier Applied Science Publishers, London, UK, pp. 249 – 261.

Tessendorf, B. A., 1991. Bacterial populations associated with Baker's yeast. Honour's Dissertation, Department of Microbiology, University of the Witwatersrand, Johannesburg, South Africa.

Tracey, R. P., van Vuuren, H. J. J. and du Toit, D. S., 1984. A method for the detection of *Issatchenkia orientalis* in a baker's yeast factory. Applied Microbiology and Biotechnology 19, 131 – 133.

Troller, J. A. (Ed.), 1983. Sanitation in food processing. Academic Press Inc., London, UK, pp. 58 – 107 and 112 – 159.

van Dam, H. W., 1986. The biotechnology of baker's yeast: Old or New Business. In: Blanshard, J. M. V., Frazier, P. J. and Galliard, T. (Eds.), Chemistry and Physics of Baking, The Royal Society of Chemistry, UK, pp. 116 – 131.

Varnam, A., 2002. *Lactobacillus*: occurrence and significance in non-dairy foods. Microbiology Today 29, 13 – 17.

Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W. and Gonzalez-Zorn, B., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clinical Microbiology Reviews 14, 584 – 640.

Viljoen, B. C. and Lues, J. F. R., 1993. The microbial populations associated with post-fermented dough and compressed baker's yeast. Food Microbiology 10, 379 – 386.

Viljoen, C. R. and von Holy, A., 1997. Microbial populations associated with commercial bread production. Journal of Basic Microbiology 37 (6), 439 – 444.

von Holy, A. and Holzapfel, W. H., 1988. The influence of extrinsic factors on the microbiological spoilage pattern of ground beef. International Journal of Food Microbiology 6, 269 – 280.

von Holy, A. and Kirschner, L. M., 1991. *In vitro* and *in situ* morphological comparison of a rope-inducing *Bacillus subtilis*. Proceedings of the Electron Microscopy Society of Southern Africa 21, 169 – 170.

Wainwright, M., 1992. An introduction to fungal biotechnology, John Wiley and Sons. West Sussex, England, UK, pp. 147 – 151.

Walter, F. G. (Ed.), 1953. The manufacture of compressed yeast, 2nd Ed., Chapman and Hall Ltd., London, UK, pp. 1 – 20 and 132 – 233.

Ward, O. P. (Ed.), 1992. Fermentation Biotechnology, John Wiley and Sons, England, UK, pp. 4 – 45 and 102 – 120.

Willcock, L., Gilbert, P., Holah, J., Wirtanen, G. and Allison, D. G., 2000. A new technique for the performance evaluation of clean-in-place disinfection of biofilms. Journal of Industrial Microbiology and Biotechnology 25, 235 – 241.

Wood, B. J. B. and Hodge, M. M., 1985. Yeast-lactic acid bacteria interactions and fermented foods In: Wood, B. B., 1985. *ibid*.

Yoshida, T., Kato, Y., Sato, M. and Hirai, K., 1998. Sources and routes of contamination of raw milk with *Listeria monocytogenes* and its control. Journal of Veterinary Medical Science 60, 1165 – 1168.

Zottola, E. A., 1991. Characterization of the attachment matrix of *Pseudomonas fragi* attached to non-porous surfaces. Biofouling 5, 37 – 55.

Zottola, E. A. and Sasahara, K. C., 1994. Microbial biofilms in the food processing industry – should they be a concern? International Journal of Food Microbiology 23, 125 – 148.