The microbial ecology of a rum production process

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This thesis is, lovingly, dedicated to

Graham Harold Fleet

“Fluffy”

Supervisor

Mentor

Friend

PLEASE TYPE

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Rum is an alcoholic beverage made from the distillate of a microbial fermentation of sugar cane molasses. Although commercial rum production started in the 16 th century, the microbial ecology of the process has remained relatively unexplored. This thesis reports an investigation of the microorganisms associated with rum production at a distillery in Queensland, Australia. Samples of raw materials (molasses, dunder, water, additives), starter cultures and fermenting molasses were systematically examined for the populations and species of yeasts and bacteria.

Molasses contained low populations (< 10^2 CFU/mL) of yeasts, *Bacillus* species and lactic acid bacteria. *Saccharomyces cerevisiae*, used as a starter culture, was the main yeast of molasses fermentation, growing to populations of about 10^7 CFU/mL. Lactic acid bacteria were consistently isolated from the molasses fermentation and reached populations of 10^7 CFU/mL. The main species isolated were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and an unidentifiable *Lactobacillus* spp. These species were indigenous contaminants within the processing environment, colonising sites that escaped effective cleaning and sanitation operations. Species of *Clostridium*, *Zymomonas* and *Propionibacterium* were not detected in the production system. Dunder, which originated from the distillation operation, was considered to be sterile, but developed a population of lactic acid bacteria (the unidentifiable *Lactobacillus* spp.) on storage. Dunder had significant concentrations of organic acids and amino acids. At concentrations of 10% and above, it significantly inhibited the growth of *S. cerevisiae* in molasses medium and to a lesser extent lactic acid bacteria. Laboratory scale molasses fermentations and distillations were performed to investigate the effect of lactic acid bacteria on the growth of *S. cerevisiae*, process efficiency and production of flavour volatiles. Both single and mixed cultures using *S. cerevisiae, L. fermentum, L. plantarum* and *Lactobacillus* spp. were undertaken. The bacteria did not restrict the growth of *S. cerevisiae* but enhanced utilization of molasses sugars and ethanol production. The work presented in this thesis is the first comprehensive and systematic study, of its type, into the microbial ecology of a rum distillery.

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Signed 

Date 20/08/15

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Victoria Green (2015)

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ABSTRACT

Rum is an alcoholic beverage made from the distillate of a microbial fermentation of sugar cane molasses. Although commercial rum production started in the 16 th century, the microbial ecology of the process has remained relatively unexplored. This thesis reports an investigation of the microorganisms associated with rum production at a distillery in Queensland, Australia. Samples of raw materials (molasses, dunder, water, additives), starter cultures and fermenting molasses were systematically examined for the populations and species of yeasts and bacteria.

Molasses contained low populations (< 10^2 CFU/mL) of yeasts, *Bacillus* species and lactic acid bacteria. *Saccharomyces cerevisiae*, used as a starter culture, was the main yeast of molasses fermentation, growing to populations of about 10^7 CFU/mL. Lactic acid bacteria were consistently isolated from the molasses fermentation and reached populations of 10^7 CFU/mL. The main species isolated were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and an unidentifiable *Lactobacillus* spp. These species were indigenous contaminants within the processing environment, colonising sites that escaped effective cleaning and sanitation operations. Species of *Clostridium*, *Zymomonas* and *Propionibacterium* were not detected in the production system. Dunder, which originated from the distillation operation, was considered to be sterile, but developed a population of lactic acid bacteria (the unidentifiable *Lactobacillus* spp.) on storage. Dunder had significant concentrations of organic acids and amino acids. At concentrations of 10% and above, it significantly inhibited the growth of *S. cerevisiae* in molasses medium and to a lesser extent lactic acid bacteria. Laboratory scale molasses fermentations and distillations were performed to investigate the effect of lactic acid bacteria on the growth of *S. cerevisiae*, process efficiency and production of flavour volatiles. Both single and mixed cultures using *S. cerevisiae, L. fermentum, L. plantarum* and *Lactobacillus* spp. were undertaken. The bacteria did not restrict the growth of *S. cerevisiae* but enhanced utilization of molasses sugars and ethanol production. The work presented in this thesis is the first comprehensive and systematic study, of its type, into the microbial ecology of a rum distillery.

CHAPTER 1 - INTRODUCTION

Rum is a distilled alcoholic beverage made from fermented sugar cane juice, sugar cane syrup or molasses (Nicol, 2003; Piggott, 2009). Most rums are produced from sugar cane molasses, although an allied product, cachaça (Faria, *et al* 2003; Rosa, *et al*, 2009), is produced in Brazil from sugar cane juice. This thesis is focused on rum produced from molasses.

Rum production evolved from the cane sugar industry, and has been produced in countries of the Caribbean and West Indies region since the 16th century. It is now produced in many other countries, including Australia. It is a significant commodity in international trade and contributes substantially to the export economies of numerous developed and developing countries. Rum represented about 8% of the global distilled beverage industry in 2011, with production of about 1.2 billion litres annually valued at about $US2.1 billion (Datamonitor, 2011; Collicutt, 2009a).

The basic process for rum production consists of the following operations: preparation of the raw material (molasses, sugar syrup or sugar cane juice); fermentation of this material; distillation of the fermented product; collection of the distillate; maturation of the distillate in wooden barrels; and packaging of the final product. Detailed descriptions of the process can be found in Lehtonen and Suomalainen (1977) and Nicol (2003), and more general overviews are given in l'Anson (1971) and Kampen (1975).

The scientific basis of rum production has been investigated and described in the literature since the early 1900s (Greig, 1885; Pairault, 1903; Allan, 1906; Ashby, 1909; Arroyo, 1945a). There has been little advance in understanding the biology of the process since that time, with particular regard to the microbiology and biotechnology involved. This contrasts with other alcoholic beverages such as beer

(Campbell, 2003), wine (Parish & Fleet, 2013) and whisky (Walker, 2012) where major advances have been made in understanding the basic biology and, now, advanced molecular biology of these processes. Current knowledge and understanding about the microbiology and biotechnology of rum production from molasses has been reviewed by Lehtonen and Suomalainen (1977), Fahrasmane and Ganou-Parfait (1998) and by Fleet and Green (2010) as part of this thesis.

Microbial fermentation is a key biotechnological process in the production of rum. The microorganisms that grow throughout this process have a major influence on the flavour and quality of the final product, and the efficiency of the overall process (Lehtonen & Suomalainen, 1977; Ganou-Parfait *et al.*, 1989; Nicol 2003). Until relatively recently, the fermentation of molasses for rum production was conducted as a traditional, spontaneous process through the growth of indigenous microflora (Fahrasmane & Ganou-Parfait, 1998). Yeasts were predominant in the process as agents of alcoholic fermentation with *Saccharomyces cerevisiae* being the main species isolated from these fermentations, but *Schizosaccharomyces pombe* was also found in some cases. Eventually, strains of *S. cerevisiae* were developed for use as starter cultures to conduct these rum fermentations (Arroyo, 1945a; Fahrasmane & Ganou-Parfait, 1998). Despite their prominent role, very little research has been done to understand the kinetics of growth of yeasts during molasses based rum fermentations and to understand how they impact on rum flavour.

Early literature (Allan, 1906; Ashby, 1907; Hall *et al*., 1935) as well as more recent literature (Ganou-Parfait, Fahrasmane & Parfait, 1987; Fahrasmane & Ganou-Parfait, 1998) also reported the association of bacteria with rum fermentations and their contribution of distinctive flavour characteristics to some products. In this context, species of *Clostridium*, *Zymomonas, Bacillus,* propionic acid bacteria and lactic acid bacteria have been mentioned. However, there appears to be no detailed studies of their growth during fermentation, how they interact with the growth of yeasts and how they impact on rum quality.

Throughout the literature, dunder is mentioned as a unique raw material used in rum production (Wustenfeld & Haeseler, 1953; l'Anson, 1971; Kampen, 1975; Wilkie *et*

al, 2000). Dunder is the liquid residue, depleted of volatile compounds, remaining in the bottom of the still at the end of distillation of the fermented molasses. It is added in various proportions back to fresh molasses medium for subsequent rum fermentation. Frequently, the product is added after it has been stored and developed an indigenous microbial flora. In these circumstances, it is thought to contribute flavour enhancing bacteria and yeasts to the fermentation process (I'Anson, 1971; Murtagh, 1999; Broom, 2003; Nicol, 2003). Despite the widespread use of dunder in rum production, very little research has been conducted to understand its microbiology or chemical composition.

The research project described in this thesis originated from an enquiry by The Bundaberg Distilling Company located in Bundaberg, Queensland, Australia. Production of rum first started at The Bundaberg Distilling Company in 1888 and it has continued to the present date. The company produces approximately 5 million litres of rum annually, valued at about $200 million dollars and accounts for 95% of the dark rum market share in Australia. In 2000, it was purchased by the multinational, alcoholic beverage company, Diageo plc.

The process for rum production at this distillery has evolved and developed somewhat empirically over the years, and the final rum product has a unique or distinctive flavour character in relation to rums, globally (Broom 2003) .The company is aware of this quality trait, but is uncertain as to what factors might lead to this property. Future development and expansion of rum production at this distillery requires a more thorough understanding of the science and technology of the overall operation, so that systems can be improved or developed to better manage production efficiency and product quality. As a basis for further development of its business, the Bundaberg Distilling Company has sought a more detailed understanding of the microbiology and biotechnology of its process. It is with this background that the microbiological investigations reported in this thesis were undertaken.

The overall objective of this thesis is to systematically investigate the microbial ecology of the rum distillery located at Bundaberg, Queensland, Australia - from raw materials (molasses, dunder, yeast and water) through yeast propagation and

fermentation. This basic information about the microbiology of the process should provide platform knowledge which the company can use to optimize its process and product quality.

Given the limited information that exists about the microbiology and biotechnology of molasses based rum fermentation, in general, it is expected the results of the thesis will have broader scientific and technological interest.

Chapter 2 of this thesis gives some background literature and information regarding the history and production of rum, the microbiology of rum production and the chemical composition of rum.
Chapter 3 reports a systematic investigation of the microbial ecology of the rum production process at the Bundaberg distillery
Chapter 4 presents a more detailed investigation of the association of lactic acid bacteria with the rum production process
Chapter 5 reports the microbiology and chemistry of dunder and examines its effect on yeast and bacterial growth during molasses fermentation.
Chapter 6 combines the significant microbiological findings of Chapters 3, 4 and 5 to conduct laboratory scale fermentations and distillations to determine the effects of various controlled microbial combinations on molasses rum fermentation.
Chapter 7 summarises important conclusions of the project with recommendations for further research and development.

CHAPTER 2 LITERATURE REVIEW

2.1 BACKGROUND

Rum is a distilled alcoholic beverage made from sugar cane juice, sugar cane syrup or molasses (Nicol, 2003; Piggott *et al*, 1995). It is often matured in barrels or vats. Many countries have defined rum within their food and beverage legislations and examples for Australia, the USA and the European Union are given in below.

In Australia, rum is defined as;

> ‘a potable alcoholic distillate…which, unless otherwise required by this Standard, contains at least 37% alcohol by volume, produced by distillation of fermented liquor derived from food sources, so as to have the taste, aroma and other characteristics generally attributable to [rum]’
>
> (FSANZ, 2000)

In the USA, rum is;

> ‘an alcoholic distillate from the fermented juice of sugar cane, sugar cane syrup, sugar cane molasses, or other sugar cane by-products, produced at less than 190° proof in such manner that the distillate possesses the taste, aroma and characteristics generally attributed to rum, and bottled at not less than 80° proof; and also includes mixtures solely of such distillates.’
>
> (USA Government, 2010)

European (EU) definition;

> ‘Rum
>
> (1) A spirit drink produced exclusively by alcoholic fermentation and distillation, either from molasses or syrup produced in the manufacture of cane sugar or from sugar cane juice itself, and distilled at less than 96% vol., so that the distillate has the discernible specific organoleptic

characteristics of rum
(2) The spirit produced exclusively by alcoholic fermentation and distillation of sugar cane juice, which has the aromatic characteristics specific to rum, and a content of volatile substances equal to or exceeding 225 g hl^{-1} of alcohol of 100% vol. (2250ppm). This spirit may be marketed with the word 'agricultural' qualifying the designation 'rum' accompanied by any of the geographical designation of the French Overseas Departments as listed in Annex II
(3) Bottled at a minimum alcoholic strength of 37.5% v/v'

(The Council of European Communities (EEC), 1989)

The origin of the word "rum" is uncertain and has been attributed to a number of sources (Clutton, 1974; Lehtonen & Suomalainen, 1977; Broom, 2003; Coulombe, 2004). There have been suggestions that the word is derived from the last part of the word "*Saccharum"* which is the taxonomic name for a sugar cane variety. Another derivation is from *rumbullion,* a British slang word meaning "great tumult or uproar". Possibilities also include; the Spanish word for 'ron', the French word for aroma, '*arôme'*, and a Dutch word for drinking glasses, "*roemer",* that sailors colloquially called "rummers". Despite the uncertain origin of the word, it was documented in 1654 within a law that was passed in Connecticut confiscating "Barbados liquors, commonly called Rum, Killdevil, or the like." As this law showed, not only was the origin of the word ambiguous but there were multiple names by which the rum beverage was known, including kill-devil, brebaje, ron, Nelson's Blood, grog, rumbullion, taffia, guildhive, Demon Water, Pirate's Drink, Barbados water and Navy Neaters. The word was also used in an order penned by the Governor-in-Council of Jamaica regarding rum and its consumption on 3rd of July 1661 (Thomson, 1885).

Rum is the product of a microbiological fermentation and this thesis is focused on the microbiology of that process. The literature review presented in this section provides some background information on the history of the rum industry and the process of rum production. This is followed by a compilation and critical evaluation of the research conducted on the microbiology of the process and how this microbiology impacts on rum quality and production efficiency. The gaps in

knowledge and needs of further research are identified and discussed. In contrast to many other alcoholic beverages, such as beer, wine, and whisky, there are only a few general discussions or reviews on the science and technology of rum production and these include Arroyo (1945a), Fahrasmane & Ganou-Parfait (1998), Lehtonen & Suomalainen (1977), Nicol (2003) and Fleet & Green (2010).

2.1.1 Origins of the Rum Industry

Discussions on the history of rum production have been given by Clutton (1974), Lehtonen & Suomalainen (1977), Nicol (2003), and Broom (2003). The origin of rum can be traced back to around 2000BC from obscure references in the sacred Hindu texts known as “The Vedas”, which refer to two beverages made from sugar cane by-products, one from molasses (gandi) and the other from cane juice (sidhu). From that time, over 3500 years ago, until the present day, rum production has been intertwined with the development and expansion of the sugar industry (Nicol, 2003; Coulombe, 2004).

Originally grown in Asia, specifically the East Indies (Indonesia and Papua New Guinea), sugarcane was taken west by trading between the Chinese and Arabs. Grown in Northern Africa by Arabs, the “sweet spice” was introduced to the western European world during the Crusades of the 11th century. Sugar was reportedly first available in England in the late 11th century. Imported to Europe from the East at great cost, there was an increasing need for the European countries to instigate production of their own supplies. By the 15th century, the Portuguese had colonised Madeira where sugarcane was successfully grown. In 1492, Columbus sailed to the Caribbean region with sugarcane cuttings, hopeful of starting a sugar industry in the Americas (Nicol, 2003). The cultivation of the cuttings was extremely successful and, on his second voyage, Columbus, bound for Cuba and Hispaniola, took more cuttings. Sugar cane cultivation quickly spread throughout the Caribbean region, including locations such as Jamaica, Martinique, Puerto Rico and Cuba, Barbados, Trinidad, Haiti, Guadeloupe, the Virgin Islands,

the Dominican Republic, Guyana and Brazil. Slaves from Africa were introduced to work on the plantations and harvest the sugarcane (Broom, 2003; Nicol, 2003; Coulombe, 2004).

Once harvested, the sugarcane was crushed and the resulting juice collected and boiled. After boiling, the liquid was allowed to cool, and crystalline sugar formed in lumps which were extracted, leaving behind a thick, dark syrup-like juice termed '*melazas*' or molasses, in English. This waste product was also sold as a sweetener because the demand for sugar outweighed its production in the 16th century. Any molasses that was unsold was usually set aside, frequently with the addition of water, reboiled and left, whereupon it underwent a natural fermentation. An alcoholic spirit was derived from the ferment by distillation and generally, was of dark colouration and relatively unpleasant in flavour. Nevertheless, it appealed to those such as the African slaves, who could not afford finer liquors and its sale returned a healthy profit to plantation owners. By the end of the 19th century, most plantations that could afford distillation equipment were producing liquor, and approximately 200 000 gallons (757 082 litres) per year was being produced on the island of Barbados alone. The distilled spirit was then, either consumed locally or, purchased by naval ships that frequented the seas nearby. The presence of these naval ships discouraged intervention by pirates and lead to a long association between the rum producers and naval companies, with the eventual export of rum to other countries, including Europe, by the end of the 17th century (Bluhm, 1983; Broom, 2003).

By the end of the 17th century the Caribbean region had become the world centre for rum production, and a thriving industry had been established. Rum had now become a global commodity. Rum's popularity drew from its stark contrast to the more traditional European style spirits such as gin and Scotch whisky. Throughout the 18th century, Colonial America produced more rum than the entire Caribbean region and the popularity of the spirit in that country as an alcoholic beverage continued until the Great Depression of the 1930's. The Depression caused sugar

and rum sales to dramatically decrease, forcing distilleries to close. The introduction of American Prohibition, prohibited the manufacture, sale and transport of alcohol in the United States from January 1920 to December 1933, also saw a decrease in overall rum production around that time. These circumstances caused distillers to form alliances to better regulate the sale and price of rum in America and the Caribbean, and set the foundations for the modern commercialisation of rum production. This cooperation of groups of distillers was the forefront of the “big brand” era that has characterized the rum industry for the last one hundred years (Broom, 2003).

World War II lead to a resurgence in Caribbean rum popularity, mainly due to American and British distilleries ceasing production of drinking spirits such as gin and whisky as they switched their activities to production of industrial alcohol for “the war effort”. Such transitions enhanced the importation of rum from the Caribbean region. Unfortunately, this popularity did not last long after the war when consumer tastes shifted from the heavy, sweet drinks towards lighter Scotch whisky varieties and the newer import of vodka, a subtly flavoured colourless spirit. This shift in public perception and taste allowed a lighter-bodied, white rum (Bacardi) to establish itself at the forefront of the rum industry. Bacardi, although established in 1862 in Cuba, became a household name in the mid 20th century worldwide. It remains the leading rum brand worldwide (see Table 2.1 in section 2.1.1.3).

2.1.1.1 The Rum Styles

From its humble beginnings as plantation waste given to slaves as a form of payment, rum has matured into a popular choice of distilled beverage worldwide. It was the first distilled beverage consumed in America and while its popularity waned for a large part of the 20th century, it has had a revival worldwide, mainly due to the production of white rum and its use in cocktails. Rum linkage to the expansion and industrialisation of the sugar industry and the former colonisation of

the Caribbean basin by different European countries has meant that rum production is a process impacted by history and international influences, leading to many regional variations. The Spanish colonies Cuba, Panama, the Dominican Republic and Puerto Rico manufactured subtle, light rums. The islands of Barbados, Bermuda, Jamaica and other British colonies islands produced a fuller tasting rum with a darker colour. French colonial islands such as Guadeloupe, Haiti, and Martinique, produced a very different type of rum (Rhum Agricole) (Kampen, 1975; Broom, 2003). This rum is generally produced from sugar cane juice rather than molasses and, consequently, exhibits a flavour closer in characteristics to the sugar cane and is usually more expensive. Cachaca is a spirit produced in Brazil from sugar cane juice, similar to rhum agricole, but it is often triple distilled for a smoother, lighter flavour (I'Anson, 1971; Clutton, 1974). Consequently, rum is a term that represents various styles of product.

2.1.1.2 History in Australia

Sugar cane was first introduced to Australia and Norfolk Island in 1788 but this tropical grass did not fare well in the southern parts of these new British colonies. The first successful crop was harvested more than 50 years later, in 1862, by Captain Louis Hope at a plantation east of Brisbane. With the introduction of regulations encouraging the production of sugar, it did not take long before sugar was a major crop (Kerr, 1983). By 1890, sugar cane was grown as far north as Mackay along the east coast of Queensland, Australia. North Queensland has since developed into a major region for Australian sugar production, producing a total of 4.75 million tonnes of sugar annually, valued at $AUD 1.75 billion. Of this, 85% is exported and sold internationally. Australia is the second biggest raw sugar producer worldwide after Brazil. Other raw sugar producing countries include India, Thailand, Guatemala, Cuba and South Africa. (Queensland Sugar Limited, 2010).

The rum industry in Australia has an interesting association with the development of the country since its establishment as a British colony in 1788. In the very early

stages, rum was imported from other colonies of the British Empire. Rum can be linked to several historical events throughout the colony. One of the most interesting events occurred during Captain Bligh's office as governor of England's southern penal colony. He was deposed by the New South Wales Corps (the "Rum Corps") because of his confrontational approach to several influential colonists who defied government regulations and engaged in private trading enterprises for personal profit. The Rum Rebellion occurred on 26 January 1808, with the NSW Corps marching on Government House and arresting Bligh for being "unfit to govern". This was the only time in the history of Australia that any government was changed by force (Fitzgerald & Hearn 1988; Bligh & Currey, 2003).

Major General Lachlan Macquarie was appointed Governor in 1810. On his arrival, Macquarie found a hospital consisting of tents and other temporary structures at The Rocks at Sydney Cove. Macquarie set about earmarking property for a new, permanent, hospital but the British Government refused to allocate funds for the building. Macquarie approached a consortium of businessmen for this funding and in exchange for building the hospital, the businessmen received the exclusive rights on rum imports (60,000 gallons/ year) to sell to colonists and convict labour. Macquarie's hospital was known as "The Rum Hospital". It still exists today as the Sydney Hospital and is the oldest hospital in Australia (Lewis, 1992).

In 1869, a man named James Stewart, bought a small boat, converted it to steam power, and installed a pot still on the deck. The *Walrus*, as it was called, also had a small sugar mill for crushing up to two tons of cane a day. Stewart obtained a distillery licence and, on 14 April 1869, the Pioneer Floating Sugar Company started moving along rivers to small sugar plantations crushing cane and producing rum from the molasses. Due to the difficulty of keeping adequate account of the Pioneer Floating Sugar Company, the licence was not renewed in 1872 by the authorities. Stewart, however, continued to operate the distillery unofficially until 1883 (Lewis, 1992).

In 1884, the price of sugar fell, however, distilleries were encouraged to continue operation by the government (mainly due to the considerable tax revenue applicable on the product). Rum was easier to transport to other parts of Australia, mainly the east coast, due to the longer shelf life compared to beer and wine. In 1888, production of rum started at a distillery in Bundaberg, Queensland. The Bundaberg Distilling Company was originally run by the operators of local sugar mills in the region, concerned with how to deal with waste molasses from sugar production (Kerr, 1983). The distillery has been closed on two separate occasions due to fires; however, it continues to run today (Kerr, 1983)

Other distilleries that have produced rum in Australia, either previously or at time of print are; Beenleigh Distillery (Beenleigh, Queensland), The Lark Distillery (Hobart, Tasmania) (www.larkdistillery.com.au), The Hoochery (Ord River, Western Australia)(www.hoochery.com.au), The Kimberley Rum Company (Swan River, Western Australia)(www.canefire.net) and small boutique distilleries nationwide.

2.1.1.3 Rum Production Statistics

Alcoholic beverage production worldwide has been estimated to exceed 120 billion litres in 2008. The total market revenue for 2011 was estimated at US $1009.7 billion (Marketline, 2012). Any estimates are deemed to be on the low side due to sale statistics being closely guarded within the industry or difficult to calculate for some countries (Piggott, 2003).

Rum represented 7.7% of the global distilled beverage industry in 2011 (Datamonitor 2011). In 2007, the rum market was valued at $US 2.1 billion in the USA, accounting for 18% of spirit sales (Drinks International, 2011). North America accounts for the highest share of global rum sales (55%). Countries in the Caribbean region remain the major producers of rum, but it is produced in other countries where sugar cane is grown, such as the Philippines, India, Brazil, Fiji and Australia. Table 2.1 shows some statistics on rum production from 2011, giving the

brand and company name along with location of production.

Table 2.1 Rum production statistics for 2011 by leading brand names (Euromonitor, 2011)

Brand	Country	Company	Sales (million 9L cases)[a]
Bacardi	Cuba (headquarters in Bermuda)	Bacardi	19.6
Tanduay	Philippines	Tanduay Distillers	18.7
McDowell's No.1 Celebration	India	United Spirits	15.6
Captain Morgan	Puerto Rico (American)	Diageo PLC	9.2
Havana Club	Cuba	Pernod Ricard	3.8
Contessa	India	Radico Khaitan	2.4
Old Cask	India	United Spirits	2.2
Old Port Rum	India	Armut Distilleries	2.0
Montilla	Brazil	Pernod Ricard	1.6
Cacique	Venezuela	Diageo PLC	1.7

[a] 9L cases are the common way of measuring volume of sales in the alcohol industry. 9L case refers to 12 bottles of 750mL.

Australian rum production is dominated by two companies. Of these, the Bundaberg Distilling Company is the market leader with estimates of $200m net sales in 2008 (Main, 2010) and a 95% share of the dark rum market (McNicoll, 2006).

Bundaberg Distilling Company's nearest competitor is Inner Circle (Lion Nathan). First produced by the Colonial Sugar Refinery (CSR) Company in 1901, Inner Circle has a worldwide reputation as a high class, quality rum. Production of this rum previously occurred off shore in Fiji; however, the recent acquisition of the old Beenleigh distillery, by Lion Nathan Ltd, has seen production return to Australia (www.innercirclerum.com.au).

2.2 THE PROCESS OF RUM PRODUCTION

The basic process of rum production consists of: preparation of the raw materials for fermentation; alcoholic fermentation; distillation of the ferment; collection of the distillate; maturation of the distillate; blending; packaging; and sale (Figure 2.1). The main raw material can be either molasses or sugar cane juice. Most rum is produced from molasses and this process will be the focus of this review. Rum production from sugar cane juice will be briefly described in Section 2.2.1. General descriptions of the process have been given by Arroyo (1942 & 1945a), Clutton (1974), Kampen (1975), Lehtonen & Suomalainen (1977), Bluhm (1983) and Nicol

(2003) and these articles can be consulted for greater detail.

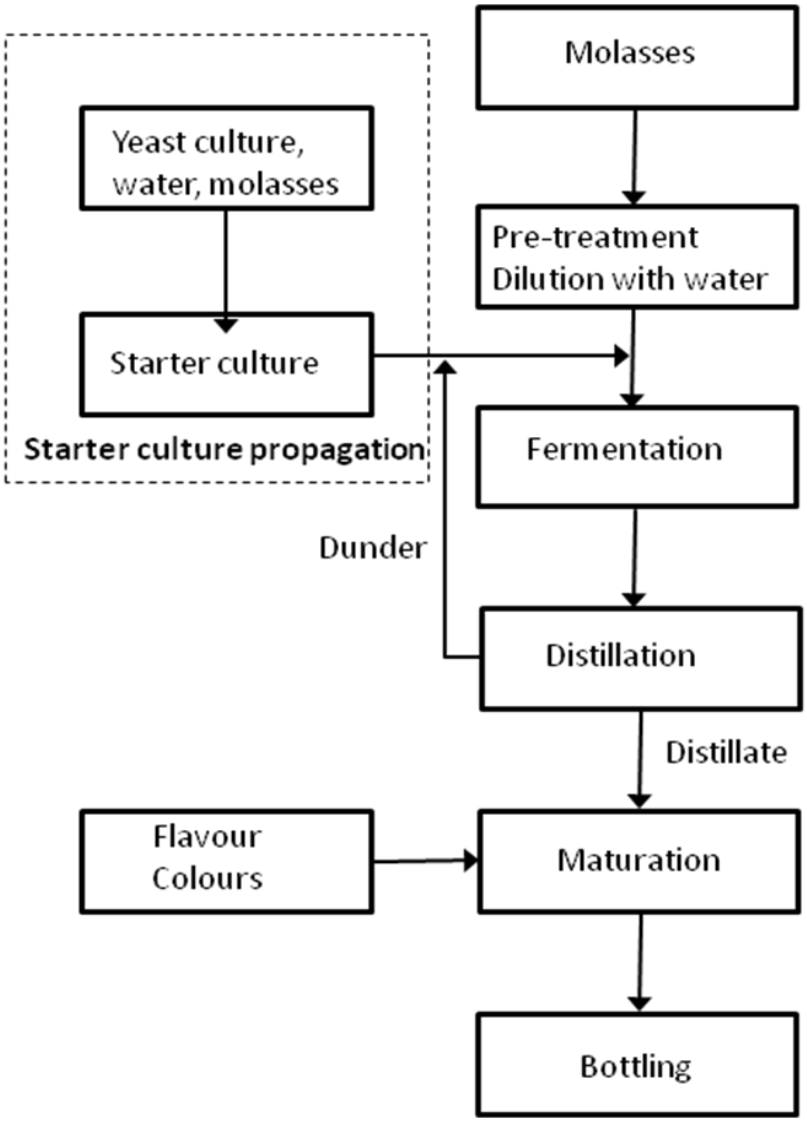


Figure 2.1 Outline of the process of rum production from molasses

2.2.1 Production

Molasses is the major by-product of the extraction of sugar from sugar producing crops. There are two main sources of molasses; cane molasses or sugar beet molasses. However, by strict definition, only sugar cane molasses can be utilised to produce rum (Kampen, 1975; Nicol, 2003).

Sugar cane (*Saccharum officinarum*) is a tall, tropical grass which has stalks that grow to heights of 2-6 metres The stems contain approximately 15% sugar (sucrose) solution but, depending on sugar cane cultivar, farming methods and factors such as climate, up to 50-60% sugar content can be present. Details on the botany of the sugar cane plant, its cultivation and its processing into sugar are given in Deerr (1921), Birch *et al* (1979) and Galloway (1989).

Sugar cane crops, today, are generally harvested by machines, whereas some years ago this was done manually by workers with machetes. It was often the practice to burn the sugar cane field just prior to harvesting to remove some of the fibrous plant material and to sanitize the soil However, this practice is now less common, and the cane is directly harvested without burning. The sugar cane juice is extracted from the harvested crops by crushing the stems and pressing into a dry mass (Nicol, 2003). The juice is boiled to reduce the water content to a desirable consistency for sugar extraction, leaving ‘first molasses’ after the initial boiling. ‘Second molasses’ is produced from boiling of the first molasses, where raw sugar begins to crystallize and separate. Blackstrap molasses is the final molasses produced from further rounds of boiling and crystal extraction of the second molasses. Detailed descriptions of sugar cane processing and molasses production are given in Lehtonen & Suomalainen (1977), Nicol (2003) and James (2008).

Due to its availability and comparatively low cost, blackstrap is the most commonly employed molasses in rum production (Lehtonen & Suomalainen 1977). Because of the additional steps involved in its production, blackstrap molasses has a more

pronounced, slightly bitter, flavour compared to first and second molasses. Some rum producers may also mix in a small portion of refinery molasses to their blackstrap stocks. Refinery molasses is produced during the refining of raw sugar to white sugar, which liberates extra molasses originally bound to the raw sugar crystals.

Rum distilleries are usually built in close proximity to a sugar mill, either directly adjacent or only a short distance away. Consequently, molasses is delivered to the rum distillery storage tanks either by pipe, still hot from the sugar mill, or by road tankers. Molasses is stored in either large concrete lined wells, stainless steel tanks or a combination of both. Due to its nature (low water content, high sugar content, low oxygen, low pH), molasses can be stored for long periods of time (up to a couple of years) with minimal processing required both prior to storage and also prior to fermentation. Thus, a supply is available throughout the entire year, regardless of when the sugar cane crushing season took place. Rum producers will usually possess multiple storage facilities, used on a rotational schedule, to avoid the use of ‘fresh’ molasses and also avoid the influences of seasonal variation and periodic supply (Fahrasmane & Ganou-Parfait, 1998).

Since the early studies of Arroyo (1947) on molasses and rum production, it has been realized that molasses quality will impact on rum quality and efficiency of the production process. Quality assurance testing of molasses for its chemical, physical and microbiological properties is now a routine part of rum production. The following section describes the key physical and chemical properties of molasses. The microbiology of molasses will be considered in a later section (Section 2.2.1.2).

2.2.1.1 Physical and Chemical Properties of Molasses

Molasses is a black, viscous liquid containing about 55% w/v total fermentable sugars, of which about 35% is sucrose and 20% is a mixture of glucose and fructose. In addition to water and these sugars, it contains small amounts of many

other compounds, such as; nitrogenous substances, phosphorus, metal ions, vitamins, gums and colloidal constituents. Table 2.2 summarizes the composition of molasses as reported by Bluhm (1983), Curtin (1973), Lehtonen and Suomalainen (1977), Nicol (2003) and others.

Table 2.2 Chemical composition[a] of blackstrap molasses

Properties	Amount	Properties	Amount
Water (%)	10-20	Sulfur (%)	0.5
Brix density	79.5-89	Sodium (%)	0.06-0.2
pH	5.3	Formic acid (%)	0.1
Total Solids (%)	75.0	Acetic acid (%)	0.2
Sucrose (%)	33.8-36.4	Aconitic acid (%)	0.8
Reducing sugar (%)	19.6-65	Lactic acid (%)	0.05
Total Sugar (%)	46.0-60.9	Malic acid (%)	detected
Total nitrogen (%)	nd-1.5	Citric acid (%)	detected
Total Fat (%)	0.0	Biotin (µg/g)	0.36-3.2
Boron, ppm	410	Chlorine (µg/g)	745.0
Iron, ppm	158-249	Pantothenic Acid (µg/g)	20-120
Manganese, ppm	35-57	Pyridoxine (µg/g)	6.5
Copper, ppm	28-36	Riboflavin (µg/g)	1.8-2.5
Zinc, ppm	10-20	Thiamine (µg/g)	0.9-8.3
Nickel, ppm	1	Inositol(µg/g)	6000
Lead, ppm	0.78	Nicotinamide (µg/g)	20-25
Cobalt, ppm	0.54	Folic Acid (µg/g)	0-0.04
Potassium (%)	2.6-5.0	Crude Protein (%)	3.0
Magnesium (%)	0.3-1.0	Gums (%)	nd-6.3
Calcium (%)	0.2-2.0	Ash (%)	8.1
Phosphorus (%)	0.08-0.2	Sulfated ash (%)	7-11.57
Chlorine (%)	1.4	Apparent purity	28.67

[a] Data obtained from Nelson & Greenleaf (1927), Arroyo (1947), Burrows (1970), Wythes *et al* (1978), Bluhm (1983), Curtin (1973), Murtagh (1995 a, b), Nicol (2003), Bortolussi & O'Neil (2006), Amorim *et al.* (2009)
nd –not detected
detected – component detected not quantified

As mentioned previously, molasses is rich in fermentable sugars and these are the main chemical components of this raw material (Table 2.2). Traditionally, distillers have used °Brix as a measurement of sugar content in molasses and molasses quality. For production of good quality rum with desirable flavours and ethanol yield, molasses with a °Brix of 87.6 has been recommended. Lesser quality rums are obtained from molasses with °Brix less than 85.4 and °Brix greater than 88.2 (Lea & Piggott, 2003). However, the °Brix value does not give an accurate correlation with total sugar content (Baker 1979; Nicol, 2003) because it is a

measure of soluble solids and molasses contains many soluble solids that are not sugars. Total fermentable sugar is easily quantified by high performance liquid chromatography (HPLC) and such measurements would be the basis for better quality grading of molasses than °Brix (Baker 1979; Nicol, 2003).

Molasses selection is critical in order to obtain efficient fermentations with high levels of ethanol, and the production of rum with a desirable flavour profile. The fermentation medium of molasses needs to contain appropriate levels of nitrogen, phosphorus, vitamins and minerals in addition to fermentable sugars (Walker, 2004).

Molasses has a pH of 5.0 to 5.5 due to the presence of numerous organic acids, the most prevalent of which are acetic, malic, lactic and citric acids. Total nitrogen represents no more than 1.5% of molasses and consists of free amino nitrogen (ammonia and amino acids) and crude protein (about 3%). The gums of molasses can constitute up to 6% and are represented by hemicelluloses, pectins and dextrins which are found in sugar cane, and levans that may be produced by bacteria during the sugar cane milling process (Schooness & Pillay, 2004). Several vitamins have been found in molasses (Table 2.2), with inositol and pantothenic acid being the most prevalent.

Many factors affect the composition and quality of molasses, and these include the soil type, ambient temperature, moisture, season of production, cultivar and cultivation of sugar cane, the sugar refining process and conditions of molasses storage (Murtagh, 1995 a, b; Bortolussi and O'Neil, 2006). Thus, variation may be found in nutrient content, flavour, colour, viscosity and total sugar content.

2.2.1.2 Molasses Properties and Microbial Growth

Molasses contains sufficient carbon and other micronutrients such as metal ions and vitamins to allow microbial growth. However, it has a very high concentration

of soluble sugars (50-60%), low water activity (Aw 0.76), low pH, and is a poor source of nitrogen and phosphorus. Consequently, it is an extremely stressful environment for microbial growth and survival (Lehtonen & Suomalainen, 1977; Kampen, 1975) and only a few, well adapted species will tolerate these conditions (see 2.4.1).

Upon dilution with water, the stresses of high sugar content and low water activity are relieved and it becomes more favourable for microbial growth. For rum production and fermentation by the distiller's strains of the yeast *Saccharomyces cerevisiae*, it is usually diluted to about 15-40 °Brix or 15-30% fermentable sugars (Lehtonen & Suomalainen, 1977). In some cases, additional supplies of nitrogen, vitamins and other micronutrients may be added to encourage growth of the distiller's yeast (Lehtonen & Suomalainen 1977; Nicol, 2003) .Fermentations lacking these nutrients tend to be slow, often unable to progress, giving a condition that is known as becoming "stuck".

Stress on the yeast cells can also cause them to produce undesirable flavours. Therefore, it is of vital importance to rum quality and production efficiency to ensure that the molasses medium contains sufficient levels of nitrogen and phosphorus (Cacho & Murphy 1988). In order to rectify these deficiencies, rum producers will often supplement the medium with ammonium phosphate or ammonium sulphate at levels between 0.03 -0.06% (w/v) (Bluhm, 1983). Commercial yeast nutrients such as Fermaid A® may also be used (www.lallemandwine.com). It is important to carefully control the addition of these supplements since excess nitrogen in the medium may inhibit yeast growth and affect production of flavour metabolites (Walker, 2004).

Molasses contains several substances that could be inhibitory to yeast growth if they are present at high concentrations. Hydroxymethyl furfural (HMF) is a product of the overheating of cane molasses during milling at a low pH. HMF levels of 0.4% can be tolerated by yeasts but higher amounts should be avoided. Several low

molecular weight organic acids can be present in molasses. Acetic acid may be found at levels of up to about 1%; however, levels of 0.25% or more are known to be inhibitory to yeasts. Butyric acid is, similarly, inhibitory to yeast at 0.1%-0.5% and may be found at levels up to 1.0%. Similarly valeric acid is inhibitory to yeast at 0.1%-0.5% and has been found at levels of 0.1% (Lehtonen & Suomalainen, 1977). Consequently, it is necessary to prepare and store molasses under conditions that prevent the development of these inhibitors.

2.2.1.3 Preparation of Molasses for Use in Fermentation

As mentioned previously, rum distillers generally store molasses in bulk quantities in wells or tanks, and draw from these supplies to prepare it for fermentation. Such preparation includes clarification, adjustment of pH, heating to inactivate microorganisms, dilution with water, addition of nutrients for yeast growth and addition of dunder (Clutton, 1974; Nicol, 2003; Lehtonen & Suomalainen, 1977; Kampen, 1975). The following sections describe the operations for preparing the molasses for fermentation.

Immediately prior to use in fermentation, molasses is clarified by a combination of chemical or physical processes to partially remove suspended solids. This is usually done by addition of flocculating agents and allowing the solids to sediment. Centrifugation may also be used to clarify the molasses. At this stage, the pH is adjusted to values around 5.0-5.5 by addition of sulphuric acid, and the mixture is given a mild heat pasteurization treatment (80°C). Duration of pasteurisation is often unrecorded and as such will vary among distilleries. It is important to remove colloidal material from the molasses, otherwise it will cause severe fouling of the distillation columns, thereby leading to inefficiency of the stills and production downtime due to increased frequency of cleaning (Murtagh 1995a).

After settling or centrifugation of the solid materials, the clarified liquid is pumped off, and then diluted with potable water to give a final concentration of 100-150

g/litre for fermentable sugars (15 to 20 °Brix). Yeast nutrients such as ammonium sulphate and vitamin mixtures may be added at this time to ensure complete fermentation (Paturau, 1989; Nicol, 2003). Also, at this stage, dunder is added to the mix. The addition of dunder is a traditional, unique part of the rum making process and, in this context, is described in the literature from studies published since the early 1900s (l'Anson, 1971; Broom, 2003; Nicol, 2003). The role of dunder and its use in rum production is discussed in more detail in a later section (Section 2.2.1.5). The diluted molasses containing added nutrients and dunder is then cooled to about 30°C by heat exchange and pumped to large vessels for fermentation.

2.2.1.4 Dilution Waters

Water is used to dilute the molasses for preparation of the fermentation medium (Arroyo, 1942 & 1945a). Waters used for dilution purposes may originate from various locations including town water, treated distillery waste waters, rivers and creeks and rainwater storage tanks. As such, the quality may vary greatly and microbial testing and chemical analysis should be undertaken. Chemical testing should determine ion content, heavy metal presence and hardness. Microbiological testing for total viable count, coliforms and *E. coli* and *Clostridium perfringens* should be done as a minimum (Fahrasmane & Ganou-Parfait, 1998). Detailed testing procedures and results are difficult to find in the rum literature as water testing has been deemed more important for dilution of the finished matured spirit rather than that used for dilution of fermentation medium. Arroyo (1945a) recognised the importance of using good quality water, during dilution of molasses, to limit the potential production of off-odours by contaminating microflora during subsequent fermentations. His research also discussed the potential of increasing the mineral content of molasses because of its effect on yeast growth and activity.

2.2.1.5 Dunder

Dunder is the liquid residue, depleted of volatile compounds, obtained from the stills after distillation of the fermented molasses (Kampen, 1975). As a result, dunder is rich in suspended solids, non volatile compounds, particularly non volatile acids, and non viable microbial biomass. Dunder is either added to the fermentation medium fresh from the primary distillation columns or after prolonged storage in dunder pits. Although the literature reports widespread use of dunder in rum production, there is a diversity of descriptions as to what it actually represents. Table 2.3 gives a summary of some of the descriptions used in the literature in reference to dunder.

Table 2.3 Definitions of dunder used for rum production

Author	Year	Definition
Wustenfeld & Haeseler,	1953	Spontaneous, bacterial soured cane molasses slops from fermentation plant. It is important for producing characteristic rum esters
I'Anson	1971	Added to fermented molasses medium. It is the lees of previous distillations which has been allowed to age and undergo bacterial fermentation. It is a strong smelling liquid consisting of high acid and ester content and causes the distillate to have a rich and fruity aroma. This aroma is characteristic of Jamaican rums.
Kampen	1975	Bottom product of a distillation column; rich in both yeast nutrients and acids
Murtagh	1995b	Old stillage that has been stored in open tanks to allow development of strong bacterial flora
Fahrasmane & Ganou-Parfait	1997	Denoted as "stillage" being the residual liquor from distillation also known as slops or "spent wash"
Wilkie *et al*	2000	Also termed distillery wastewater, distillery pot ale, distillery slops, distillery spent wash, dunder. Mosto, vinasse and thin stillage The aqueous by-product from the distillation of ethanol following fermentation of carbohydrates.
Broom	2003	The acidic spent lees (the non-alcoholic residue left in the still after distillation) which has been aged outside in dunder pits to concentrate the acetic/butyric acids and the ester content
Nicol	2003	Residue of wash distillations and is allowed to ferment naturally in a "dunder pit". It is a naturally developed inoculum for fermentations containing wild yeasts and anaerobic bacteria.
Australian Government	2006	Sugar cane lees used to promote fermentation of rum. Residue left in the still after distillation and is generally used in the process of slow fermentation
Melamane, Strong & Burgess	2007	Also known as distillery wastewater, stillage, distillery pot ale, distillery slops, distillery spent wash, mosto, vinasse, thin stillage Aqueous by-product of the distillation of ethanol, wine and some waste biological material

There appears to be no standard procedure for the use of dunder in rum production. It is mostly added to molasses fermentations at the time molasses is diluted with water. If dunder is added directly from the stills, it is considered to be sterile because of its prior heat treatment in the stills. However, it is the practice with some rum producers to store the dunder until needed in covered wells (Olbrich, 1963; l'Anson, 1971; Murtagh, 1995b; Broom, 2003). During storage, a complex microflora of yeast and bacteria are likely to grow within the dunder and, therefore, will impact on the microbial ecology of the molasses fermentation and, consequently, rum flavour.

The amount of dunder added to the molasses varies with the rum producer and can range from 0- 50% (l'Anson, 1971; Fahrasmane & Ganou-Parfait, 1998) but mostly it is added at about 0-10%. The reasons for its use are also varied and include; lowering molasses pH, providing an added source of nutrients for microorganisms that conduct molasses fermentation, recycling part of the process water to minimise water usage and disposal costs, and providing a source of wild yeasts and bacteria for molasses fermentation, if stored dunder is used (Olbrich, 1963; l'Anson, 1971).

Lowering the pH is thought to encourage the growth of *Schizosaccharomyces* yeasts in comparison to *Saccharomyces* yeasts during fermentation, and additional nutrients from the dunder may encourage the growth of bacterial species that could favourably impact on rum flavours (Kampen, 1975; Faharasmane & Ganou-Parfait, 1998).

Despite the use of dunder in rum production and its potential impact on product quality and process efficiency, there is little published information on its microbiological and chemical properties. Essentially, dunder is a microbial (mostly yeasts) culture that has been heated to 80-110°C for an extended period. Volatile metabolites would have been driven off, leaving a concentration of non-volatile metabolites (e.g. non-volatile acids). When examined under the microscope, it shows masses of yeast and bacterial cells, along with other particulate debris

(Kampen, 1975). Theoretically, the microbial cells should be dead due to the high temperature of distillation. The heating process would have extracted and partially degraded the proteins, lipids, polysaccharides and nucleic acids of these microorganisms. However, if the dunder is subsequently stored before use in rum fermentations, it is likely to become contaminated and support the growth of a specific microbiological flora, as already mentioned. It is broadly mentioned throughout the literature that dunder is acidic (with high levels of butyric and acetic acid) and is enriched in nutrients such as amino acids, vitamins and peptides (Nicol, 2003).

Table 2.4 is an accumulation of data from articles describing the properties and composition of dunder (or distillery wastewater). As limited information is available, there are large variations in ranges for some of the chemical properties listed.

Table 2.4 Chemical properties of dunder (distillery wastewater molasses fermentations)

Properties	Value	Properties	Value
pH[a,c]	3.0-5.0	Protein (w/w) [d*]	21.0
Electrical conductivity	346	Methionine (w/w) [d*]	4
Volatile fatty acids (g/L)	1.6	Tryptophan (w/w) [d*]	2.4
BOD (g/L)[a,c]	8.7- 48	Lysine (w/w) [d*]	10
Total chemical oxygen demand(g/L)[a,b,c]	51.2-120	Calcium (w/w) [b,d*]	1.46-3
Volatile solids (g/L)	50	Iron (w/w) [d*]	0.35
Volatile suspended solids (g/L)[b]	1.8-2.8	Phosphorus (w/w) [d*]	0.23
Total solids (g/L)[a]	28.1-111	Phenol (w/w) [d*]	0.23
Total nitrogen (g/L)[a,c]	0.6-2.2	Crude Fibre (w/w) [d*]	5.5
Suspended solids (g/L)[a]	1.0-7.0	Ash content (w/w) [d*]	56
Nitrates (g/L)	1.5- 4.9	Glucan (w/w) [d*]	3.6
Calcium (g/L)[a]	0.6-2.6	Mannan (w/w) [d*]	2.8
Magnesium (MgO) (g/L)[a,b]	0.15-1.1	Glycogen (w/w) [d*]	1.2
Sulphate (g/L)[a,b]	1.3 – 5.2	Thiamine (w/w) [d*]	0.06
Reducing sugar (g/L)[a]	1.0-15.0	Ascorbic Acid (w/w) [d*]	3
Ammonia (mg/L) [a]	40-200	Acetic Acid (g/L)[b]	1.34-1.6
Potassium (K2O) (g/L) [a,b]	nd-20.0	Propionic Acid (g/L)[b]	0.09-0.12
Iron (mg/L) [a]	120	Butyric Acid (g/L)[b]	0.29-0.66
Copper (mg/L) [a]	3.8		

[a] Basu (1975); [b] Bories *et al* (1988); [c] Wilke *et al* (2000); [d] Rameshwari & Karthikeyan (2005) ([*]composition given as percentage dried yeast sludge)

2.2.2 Preparation of Fermentation Media

2.2.2.1 Yeast Inoculum for Fermentation

The microbiology of rum fermentation is discussed later in Section 2.4. Modern rum distilleries conduct the fermentation by inoculation with starter cultures of selected strains of the yeast, *Saccharomyces cerevisiae*. These strains may be maintained as pure cultures “in house” and propagated to inoculum volumes as needed on site. Some distillers may purchase their yeast as active dry cultures from specialized companies and rehydrate them for direct inoculation into the fermenters according to the manufacturer’s instructions. Companies that supply distiller’s yeasts for rum production are Lallemand (www.lallemand.com) and AB mauri (www.abmauri.com). The process for yeast propagation on site is briefly outlined here and described in Murtagh (1995 a) and Nicol (2003).

Yeast propagation is initiated by preparing an inoculum from a stock culture of the selected yeast strain. This stock culture will be maintained on site and securely stored in established culture collections for retrieval as needed. Purity of the culture is verified by agar plating, from which a small volume (100 – 500 mL) of liquid culture is prepared under strict aseptic conditions. This culture is used to inoculate about fifty litres of sterile medium (autoclaved) and incubated with aeration to increase the numbers of growing cells prior to aseptic transfer of this culture to a larger volume (500 litres). Culture transfer to progressively larger volumes is conducted to provide a yeast inoculum that gives 10-30% of the final molasses fermentation and a starting yeast population of approximately 10^6 cells/mL (Murtagh, 1995a).

The propagation medium is usually similar to the molasses fermentation medium (with yeast nutrient addition) to ensure that the yeast is well adapted to that condition. While medium used in the early stages of propagation can be sterilized by autoclaving, the final stages of propagation usually require large volumes of molasses medium that is not autoclaved and has been processed in a manner

similar to the final molasses fermentation medium to contain about 10-15% fermentable sugars (Murtagh 1995b, Nicol 2003).

Glucoamylase and possibly yeast foods are also added to the propagation medium; however, few guidelines are available. The temperature at which propagation is conducted is normally monitored and controlled. There are variations in temperature from plant to plant (depending on yeast strain). Generally, propagation is conducted at a temperature at least 2-5°C below that of normal fermentation temperature. Once the yeasts have entered their active stage of growth (log phase), they are transferred from the propagator into the batch fermenter. There are typically 4 different propagation systems that can be used in industry; continuous, semicontinuous, multiple batch and single batch (Nicol, 2003).

Throughout propagation, quality control measures should ensure cell viability and culture purity. Yeast cell viability can be quickly determined using methylene blue (or other cellular stain) to differentiate between viable and non-viable cells. Counts performed in combination with a haemocytometer can give relatively quick approximations compared to cultural plating methods. Cultural purity can also be quickly determined using microscopy; however, cultural plating or real time PCR can also be used (Simpson, 1973; Nicol, 2003)

2.2.2.2 Fermentation

Fermentation by microorganisms, principally yeasts, is the key operation in rum production. The profile of flavour volatiles that distinguish rum from other distilled alcoholic beverages is produced during the fermentation of molasses by the microorganisms that grow. Without this fermentation, there would be no rum. Yeasts conduct an alcoholic fermentation of the sugars in molasses, metabolizing them into mainly ethanol and carbon dioxide, and a vast array of small amounts of secondary end products. In some cases, bacteria may be associated with the

fermentation (Lehtonen & Suomalainen, 1977; Ganou-Parfait *et al* 1991.; Fahrasmane & Ganou-Parfait, 1998). The microbiology and biochemistry of the fermentation will be discussed in more detail in Section 2.4.

In earlier times, fermentation was conducted in large, open concrete tanks or large wooden vessels. Today, most rum fermentations are conducted in large (up to 100,000L) stainless steel closed “cyclindro-conical” vessels. These vessels are equipped with stirring and sparging devices, temperature control, and cleaning in place (CIP) facilities (l’Anson, 1971; Nicol, 2003; Broom, 2003; Piggott, 2009). Although the fermentations may be gently stirred to keep the yeast cells in suspension, they are not aerated.

Rum fermentations can vary in length from 24 hours up to 10 days. Most distilleries run fermentations to a standardised time for each specific rum. Longer fermentations are used to produce the heavier flavoured rums, while shorter fermentations (24 -30 h) are used to produce lighter style rums. Fermentations are generally conducted at 28-35°C to maximize their rate of completion, and are considered complete when the desired alcohol levels (%) have been reached (approximately 5-7%). Some distilleries use change in final gravity or ° Brix, from set up to determine the completion of fermentation; however, it should be noted that all three units are related (Destruhaut *et al*, 1985; Fahrasmane & Ganou-Parfait, 1998).

Since the fermentation process generates heat, it is necessary to cool the fermenters so the temperature does not exceed 37°C. At temperatures exceeding this value, the yeast becomes sensitive to the increasing levels of ethanol and may be inactivated. If this occurs, the fermentation will stop and remain incomplete or “stuck” (Arroyo, 1945a; l’Anson, 1971). Such occurrences lead to major inefficiencies. Temperature control is needed to ensure that the temperature limits are not violated and that yeasts are not killed in the process. Cooling can be provided to help control temperature fluctuations. This can be in the form of;

internal cooling coils or panels, double jacketed walls with cooling in the outer walls, recirculating spirals, plate and frame or shell in tube heat exchangers (I'Anson, 1971; Broom, 2003)

2.2.3 Distillation of Ferment

Distillation is a critical process in rum production that separates, concentrates and selects the volatile components of the fermented molasses (Bluhm, 1983). The volatile fraction of the fermented molasses consists predominately of ethanol, and lesser amounts of higher alcohols, organic acids, esters, phenols and some carbonyl and nitrogenous compounds. The use of the collective term "congeners" has been applied to describe all volatile components of rum other than ethanol (Nykanen & Suomalainen, 1983; Murtagh, 1995 a, b).

Distillation is used to capture most of the ethanol and refine flavour by selecting for the types and concentrations of other, desirable volatile compounds; however, it does not create these base components. Creation of the desirable flavour volatiles occurs mainly during the fermentation of molasses, but some may occur in the molasses before fermentation (Yokota & Fagerson, 1971). Distillation can produce new compounds via esterification, dehydration etc from the base components produced in fermentation. The use of pot distillation (outlined in Section 2.2.3.1) is known to increase furfural concentration (Madrera *et al*, 2003).

The technology for the commercial distillation of alcoholic beverages, including rums, varies with the manufacturer but the general principles of the process are common and are described in Piggott (2009a, b). Some other reviews and discussions of the distillation process as it relates to rum production are given by Murtagh (1995a), Reche *et al* (2007) and Sampaio *et al* (2008).

Distillation is based on the principle that different components within a liquid mixture, such as the fermented molasses, have different temperatures at which

they boil and transform to a vapour or gaseous phase. Heat is applied to the liquid mixture. Simply, the smaller, more volatile components are vaporised and boil off first and are progressively followed by the less volatile components. Non-volatile substances are left in the liquid mixture. As the temperature of the vaporised fraction is decreased, the individual components revert to their liquid phase and can then be collected in this state. This process of transformation back to the liquid phase is called condensation (Wankat, 2007).

The basic apparatus for distillation consists of a vessel (still) in which the liquid mixture is heated. The base of the vessel is attached or connected to columns, into which the volatiles vaporize and eventually condense back to a liquid, and capture vessels for collecting the condensed liquid (distillate). The columns may be differentially cooled to encourage condensation (Kampen, 1975; Nicol, 2003).

Distillation efficiency can be increased by the addition of a reflux step. This is a method of returning a proportion of the condensed distillate back into the distillation column. The down flowing reflux liquid enters the column and cools. It condenses the rising vapours and works to increase the separation efficiency of the distillation column. Increasing the amount of reflux for a column will improve the separation of lower boiling components from higher boiling compounds, resulting in a distillate with a higher composition of a desired product (Wankat, 2007).

Separation of the volatile compounds is based on volatility differences and occurs through heating (and cooling). The degree of separation of the desired component may be affected by various operating conditions such as pressure, temperature, the initial feed composition and liquid phase conditions. Controlling the distillation process is crucial for the production of product with consistent and desirable quality (Nicol, 2003; Wankat, 2007).

Two types of distillation processes are used in the production of rum: batch distillation and continuous distillation. Batch distillation, in pot stills, is used to

manufacture rums with stronger, heavier flavours such as those of Barbados, Bermuda, Jamaica and other English speaking regions in the Caribbean. Continuous distillation, using column stills, is used for the production of lighter style rums of the former Spanish colonies (such as Cuba, Panama). Some distilleries use a combination of both techniques (l'Anson, 1971; Clutton, 1974; Lehtonen & Suomalainen, 1977; Bamforth, 2007; Sampaio *et al* 2008).

2.2.3.1 Batch Distillation

Pot stills are the earliest known distillation apparatus and, until the mid 1800s, all rums were produced by this process. Pot stills consist of three parts; the kettle (boiler), condenser and gooseneck, similar to those used in whisky distillation (see Figure 2.2 and Figure 2.3(a)). The kettle is the base of the vessel into which fermented molasses is transferred and heated by steam injection, either directly or indirectly through heating coils. Low boiling components, including ethanol, will begin to vaporise and pass through the gooseneck into the condenser (or retort depending on still). Limited reference is made to specific temperatures throughout the literature. This may have been influenced by two factors. Traditionally pot stills are heated by steam or fire, making temperature difficult to control. Distillers wishing to keep production practices secret from competitors may also have some bearing on the lack of records (Piggott, 2009 a, b).

The liquid distillate obtained from this type of process is also known as "single distillate" since it is processed through the still only once, giving a product of about 40-60% alcohol by volume (Nicol, 2003). This process gives a heavy pot still rum. Typically, however, this liquid is processed a second time, thus producing a double distillate which is cleaner and stronger than the single distillate. This re-distillation enables further separation of the desirable volatile compounds. This occurs due to the increased ethanol concentration of the primary distillate compared to the molasses fermentation. This increase in ethanol concentration decreases the boiling temperatures, thus ensuring greater variation as to when different volatile

compounds are liberated into the vapour phase. The process can be repeated several times, thereby obtaining a cleaner, stronger more rectified spirit each time. Distillation is performed batch by batch and is very labour intensive. Pot distillation is usually performed in conjunction with the addition of dunder in the fermentation, thus producing a heavy, high ester rum (Nicol, 2003; Piggott, 2009a, b).

The first fractions (first 5 minutes of process) to be collected as distillate contain about 88% alcohol by volume (ABV), but also contain some pungent less desirable flavour volatiles. This fraction, often referred to as the first cut or low wines, may be discarded. The final fractions of batch distillation will contain much less ethanol (less than 40-45% ABV) as it has already been distilled out, along with other volatiles with less desirable flavour attributes. Such fractions are often referred to as late cuts, talls or feints, and may also be discarded. The “centre” fraction (also called “hearts” or “spirit” or “middles”) usually contains the most desirable flavour volatiles and is the cut that is collected (85% ABV at the beginning). As distillation proceeds, the concentration of ethanol in the distillate decreases. Generally, collection of the “hearts” is stopped when the alcohol content of this fraction is about 40-43% ABV (Nicol, 2003; Piggott, 2009a)

Figure 2.2 Pot stills used in the production of distilled spirit. (a) crude rum still (Artisan Distiller, 2011) (b) Scotch whisky production at Roseisle distillery, Scotland. (Sword 2010)

2.2.3.2 Continuous Distillation

Continuous distillation gives a distillate with more consistent composition than batch distillation. Detailed history and theory of continuous distillation can be found in l'Anson (1971), Nicol (1989, 1993), Murtagh (1995a), and Wankat (2007 – Chapter 3). The distillation column or tower consists of two sections (see Figure 2.3(b)). The portion of the tower above the molasses feed entry point is defined as the 'rectifying section' of the tower. The part of the tower below the feed entry point is referred to as the 'stripping section' of the tower. Throughout the column there are a number of horizontal trays placed at different levels. Pre-heated fermented liquid is usually introduced at the top (or at least half way up the column).

As the liquid makes its way down the column, it is heated by rising vapour. This liquid-vapour contact occurs on the horizontal trays which commonly have holes punched through the metal, or specialised "bubble caps" which also allow for liquid-vapour contact. As the liquid flows on to the tray, the rising vapour is forced to come in contact with it. During this contact, heat is exchanged and the more volatile components tend to concentrate into the vapour. After repetitive liquid-vapour contacts over the height of the column, the most volatile compounds rise to the top of the column. This partial separation allows column distillation units to be more efficient at separating fermentation components than pot stills. Once the fermented medium reaches the bottom of the still, it contains no alcohol and is removed, as dunder, through a release valve. Careful control of the heating rate of the column, and the degree of reflux in the rectifying section, allows column operators to dictate the ethanol concentration of the primary distillate taken from the column (l'Anson, 1971; Murtagh, 1995b; Wankat, 2007). The highest temperature (usually in excess of 90°C - 95°C) in the tower will occur at the base, and the temperature in the tower will regularly and progressively decrease from the bottom to the top of the tower. To produce the temperature variations, reboilers (heat exchangers) are often used to heat and partially vaporise the liquid streams in the lower sections of the column (l'Anson 1971; Wankat, 2007).

The rectifying sections of both pot stills and continuous columns are often under reflux, which is where condensed liquid collecting near the base is continually pumped and fed back into the rectifying section near the top. It then simply combines with the liquid phase flowing down through the column. This enhances the interaction of the vapour and liquid phases and achieves greater separation of the volatile components. Distillation systems for rum production usually operate as two separate processes: primary distillation and secondary distillation. (Wankat, 2007).

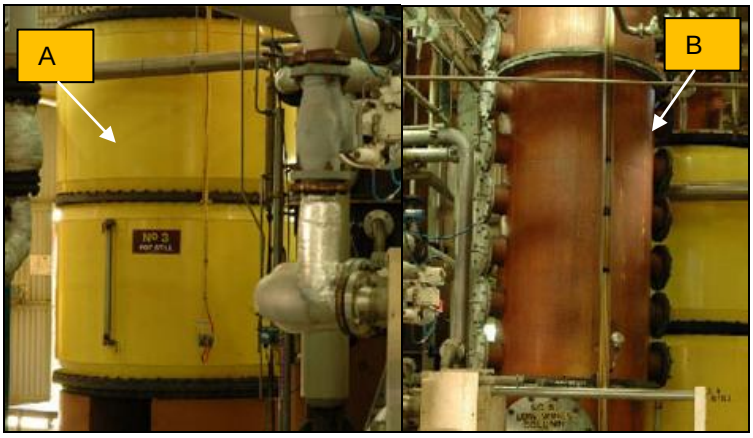


Figure 2.3 Distillation at The Bundaberg Distilling Company, Bundaberg, Australia. (a) Pot still, (b) column still. (Photo D. Flett)

Primary distillation operates similarly to pot distillation, generally referred to as “low wines” with a concentration of approximately 50% ABV. Primary distillation allows subsequent distillations (secondary distillation) to achieve greater separation of the volatile compounds. Unlike primary distillation, the distillate obtained from the secondary distillation is collected in fractions. These fractions are either based on the collection time or the ethanol concentration of the distillate collected from the rectifying section. If sufficient separation of the volatile components is achieved, each fraction should contain significantly different flavour profiles. The first fraction, rich in highly volatile components, is called the heads or ‘high feints’ and is usually discarded because it contains high concentrations of aromatic esters and acids. The second fraction is the final rum distillate or ‘raw rum’ or ‘hearts’ which is only

collected up to a certain point, once again based on either collection time or percentage of ethanol, depending on distiller. Collection of distillate beyond this point is undesirable as fusel oils constitute a large proportion of the less volatile components (I'Anson 1971; Nicol 2003; Wankat, 2007).

2.2.4 Ageing and Maturation

Freshly produced rum distillate has some strong, raw flavours that are not appreciated by all consumers. Consequently, the fresh distillate can be subjected to a process of maturation where it is stored in large wooden barrels, generally made of oak, that hold approximately 120 – 150 L (Quesada Granados *et al*, 2002). It is important to note that some provincially produced rums are not subject to ageing and are available for consumption immediately after distillation (Broom, 2003). During maturation, a range of physical and chemical interactions takes place between the barrel wood, the surrounding atmosphere and the maturing spirit. These interactions transform both the flavour and composition of the alcoholic beverage. The effects and time required for maturation are variable and are influenced by a wide range of factors, particularly the type of barrel used (Kampen, 1975; Nicol, 2003).

Maturation is not the same as ageing. Maturation is the end stage which is reached after ageing (the means to obtain the maturation). A mature rum is not defined as one that has spent a fixed period of time in a barrel. Rather, maturity is measured by the rum possessing distinctive characteristics acquired during ageing. Such characteristics are body, colour, aroma and taste. Distillate straight from a still is clear but maturation in wooden barrels gives the finished rum its yellow/golden colour, depending on how long it is aged. Ageing is simply the time the rum is stored in the wooden barrel (Nicol, 2003).

While there have been extensive studies done on the ageing of distilled alcoholic beverages such as whisky and brandy (Mosedale, 1995; Singleton, 1995;

Mosedale & Puech, 1998), there are few reports on rum maturation (de Torres *et al*, 1987; Thompson, 2009). Similar to whisky and brandy, the constituents of rum continually undergo changes during storage in wood.

Figure 2.4 shows the diversity of chemical reactions that may occur during maturation. These include: (i) direct extraction of chemical constituents from the wood, (ii) decomposition of oak on a molecular level and interaction of resulting compounds with the distillate, (iii) reactions between the constituents extracted from the wood and those in the rum distillate, (iv) reaction between wood compounds within the raw rum, (v) reactions between raw rum compounds, (vi) evaporation of volatile compounds through the cask, and (vii) interaction between the raw spirit and air present in the cask/vat. (Mosedale & Puech, 1998)

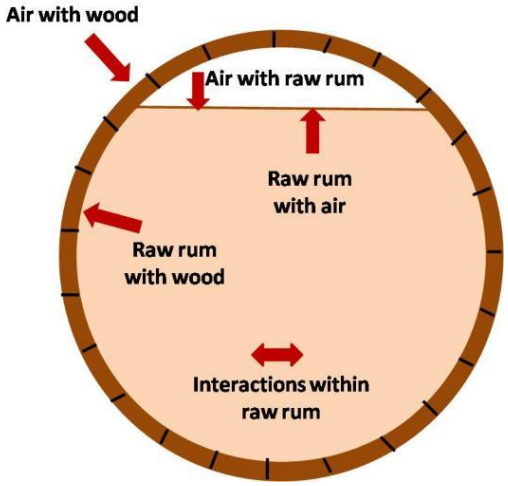


Figure 2.4 Interactions occurring during maturation of raw rum spirit in an oak barrel

The length of time that raw rum is aged depends on the type of rum being produced and also, to a certain extent, the market in which the rum will be sold. “White” rums, those that are clear and used as the basis for cocktails, are generally not aged for extended periods, except for where the law requires a minimum ageing period. These rums are usually aged in old, well used barrels and are submitted to charcoal filtering to remove any colour prior to bottling. Amber and dark rums are typically aged for a period of time between 12 months and 25+ years. Typically the longer the rum is in oak, the darker the rum (Broom, 2003;

Piggott, 2009b).

The rum industry uses two main types of barrels: those which have already been used in curing whisky and new oak barrels. The main reasons for using pre-used whisky barrels is that they are cheaper than new barrels and they have previously been ‘cured’ or charred.

Specific types of wood are used in barrel construction but American oak (*Quercus alba*) is common. It is known to produce specific flavours including vanilla, coconut and spice (Mosedale & Puech, 1998; Quesada Granados *et al,* 2002). Cask/barrel manufacture is not standardised, with large discrepancies between American and European cooperages. Barrels are very expensive and in high demand, consequently, rum distilleries may buy old barrels that have been used for the maturation of other alcoholic beverages; such as whisky, wine and brandy. Barrels are heated or charred (burning of the inside surface of the barrel) prior to raw spirit being stored. This charring changes the physical and chemical composition of the wood by caramelising sugars, increasing vanillins and helping to extract tannins (Mosedale, 1995; Mosedale & Puech, 1998; Broom, 2003).

The quality of ageing barrels is a very important parameter in the production of rum. Distillers, however, have little control over some of the factors affecting this quality such as: character of the soil and climate in which the trees were grown, age of the trees when cut, manner in which they were cut, part of tree from which the staves were derived, variety and amount of resins present, and period of ageing given to the boards prior to barrel construction (Mosedale, 1995).

Many countries have legislation governing the minimum ageing time which must occur prior to a product being sold as “rum”. For example, to be sold in Australia, the Dominican Republic and Panama, rum must be aged for a minimum of 2 years. Mexico legislation requires a minimum of 8 months. However, some countries are less stringent and allow rum to be sold without any ageing such as *rhum agricole*

and Brazilian cachaça (Broom 2003).

2.2.5 Cachaça

By definition, cachaça is a type of rum, as it is produced from sugar cane juice (Marini *et al,* 2009). Cachaça is made from fermented sugar cane juice. It is the national drink of Brazil with approximately 1.5 billion litres consumed annually (2007) with approximately 1% exported. Economically it is of great importance in Brazil, generating approximately 400,000 jobs and second only to beer in consumption (Souza, 2010). Consumption is concentrated mainly within the low-income population.

As with other rums, cachaça production consists of fermentation, distilling and ageing. While production has traditionally been artisanal, there has been a move towards more industrial production in recent years. National surveys have shown that a greater export market may be achievable if quality of the finished product were to be improved (Souza, 2010).

Few other spirits have the post distillation sensory quality that cachaça possesses: consequently, cachaça is rarely aged. This is mainly due to the target market discussed previously, and need for a cheaper product. Distinction between unaged (white) and aged (gold) cachaça is similar to other distilled beverages, with improvements with flavour mellowing and typical subtle woody and vanillin notes developing along with gold-yellow colouration during ageing (Broom, 2003; Marini *et al*, 2009)

White cachaça is usually bottled immediately after distillation (sometimes aged for up to 12 months). Gold cachaça is aged in wooden barrels and should be consumed straight or ‘neat’. Ageing is usually undertaken until 3 years; however, there are some premium cachaça that are aged for 15 years (Souza, 2010).

Changes in distillation techniques such as moving from single distillations to double distillations, as used in molasses rum production, are currently being undertaken in Brazil (Souza, 2010). Closer examination of the microflora involved with the fermentation and their effects on cachaça quality have also been conducted by some groups (Schwan *et al*, 2001; Dato *et al*, 2005; Gomes *et al*, 2010) and are discussed in more detail in Section 2.4.5.1.

2.2.6 Quality Assurance and Control in Rum Production

As a finished product, rum needs to meet the acceptance criteria of consumers. Such criteria will be determined by appearance, flavour and aroma. The rum should be free of technical faults and meet any technical and legal specifications. In addition to these criteria, there needs to be consistency in quality, which would require little variation from batch to batch production.

The basic principles of good manufacturing practice and quality assurance and control as applied to food and beverages in general, would also apply to the rum industry. General descriptions of these principles, including the Hazard Analysis and Critical Control Point (HACCP) concept are given in Jouve (2000), Bernard & Scott (2007) and Jukes (2009). HACCP is now a key component of good manufacturing practice. Although HACCP has been more broadly applied to the management of safety issues in food and beverage production, it can also be adapted to quality management.

The seven principles of HACCP are listed in Table 2.5 and these principles should be applied to the entire rum production process. There is very little published literature on the systematic application of quality management systems to rum production.

Table 2.5 HACCP principles (Bernard & Scott; 2007)

Seven Principles of HACCP
(i) Conduct a hazard analysis
(ii) Determine the Critical Control Points (CCPs)
(iii) Establish critical limits for each control point
(iv) Establish a method of monitoring CCPs
(v) Establish corrective actions
(vi) Establish procedures to verify effectiveness of HACCP system
(vii) Establish documentation and recording procedures

Connolly (1997) has mentioned the general importance of quality management in the production of distilled alcoholic beverages. With respect to rum, Nicol (2003) has listed specific operations in the process where some control is needed and these are:

- quality of molasses as obtained from the supplier,
- molasses storage,
- preparation of molasses,
- preparation of inoculum cultures for fermentation,
- control of fermentation process,
- control of the distillation process,
- management of ageing/maturation,
- packaging,
- application of effective cleaning and sanitation throughout production process.

For each of these steps in the rum production chain, it is necessary to identify and develop specific management criteria. This would require, for example, the development of specifications for all raw materials, identification of any hazards along the production chain that may impact on quality, the determination of critical control points and control limits, description of cleaning and sanitation procedures, and systematic documentation of the management plan. A detailed discussion of quality management for a rum production process will be given in Chapter 3.

2.3 Chemistry of Rum Flavour and Quality

The distinctive feature of rum and its main quality criterion is attributed to its flavour. As mentioned previously, this flavour is determined by the diversity and concentration of volatile products collected as a consequence of distillation of the fermented molasses. The main component of rum is ethanol and its concentration during fermentation reaches a maximum of 6-8% v/v. After distillation and bottling, the final concentration can vary between 37 and 80 mg/ ml (i.e. 37.0- 80.0 %), depending on brand (Nicol, 2003).The remaining components represent the congeners of which about 100 have been identified. These products have their origin as components of the molasses, components produced by metabolism of molasses by microorganisms and the changes which occur to them during maturation.

The chemistry of rum flavour has been studied since the early 1900s. Lehtonen and Suomalainen (1977) have given the most comprehensive discussion of the chemical constituents of rum flavour (aroma) and the factors that affect their production. More general discussions are provided in Clutton (1974) and Nicol (2003). Rums are categorized as heavy, light or medium style products according to their flavour or aroma profile, this being determined by the microbiology of the fermentation process and the distillation process (Lehtonen and Suomalainen, 1977). A comprehensive summary of chemical compounds found in rums is included as Appendix A.

Early studies on the chemistry of rum flavour using “classical” analytical methods, such as functional group analysis, established the presence of various volatile acids, esters, aldehydes, higher alcohols and furfurals (Simmonds, 1919; Arroyo 1942; Arroyo, 1945a). The most significant advances in understanding the chemistry of rum flavour came with the development and application of gas chromatography and later gas chromatography linked with mass spectrometry (GC-MS) or high performance liquid chromatography (HPLC) (Allan, 1972;

Cardoso *et al*, 2003).

The first study on a heavy-body rum by gas chromatography combined with infrared and mass spectrometry was done by Maarse & ten Noever de Brauw (1966) with approximately 75 flavour compounds being identified. The main components were esters, aldehydes, acetals and alcohols. The authors noted surprise at the large proportion of esters and acetals. However, there was concern that the acetals may not have been present in the rum itself, but developed as a result of sample preparation. Propanol, isobutanol and isoamylalcohol were the main alcohols present, aside from ethanol. Furans, heterocyclic organic compounds consisting of a five membered aromatic ring, were expected to be detected; however, there was interest in one specific furan, 2-methyl-tetrahydrofuran-3-one, which at the time, was also discovered as an aromatic compound present in coffee.

Nykanen (1986) studied the fatty acids of whisky, cognac, brandy and rum. Rum was found to contain the largest amount of volatile acids, being in the order of 600mg/L. Between 75% and 90% of this amount was due to acetic acid. Rum was also found to contain more butyric and propionic acid than whisky or cognac, with propionic acid predominating. The principal higher fatty acids present were myristic, palmitic and palmitoleic.

Liebich *et al.* (1970) identified over 200 flavour compounds in a Jamaican rum using GC-MS techniques. The compounds identified were characterised as various esters, acids, alcohols, phenols, lactones, carbonyl compounds, acetals, pyrazine derivatives and hydrocarbons. As a part of this research, the development of an imitation rum was completed, with the best imitation using as many of the 200 volatile components available. It was concluded that all 200 compounds were needed for a good, full rum flavour.

Pino *et al* (2002, 2007, 2012) have performed research on aroma compounds in

rums since 2002. Initial studies (Pino *et al* 2002) determined fatty acid ethyl esters in Cuban rums. Using headspace solid phase microextraction (HS-SPME), this preliminary work was developed further with the recovery of 184 volatile compounds from six different samples of four different brands of Cuban rum (Pino *et al* 2007). The volatile compounds included 64 esters, 47 benzoic compounds, 16 terpenoids, 14 alcohols, 10 acetals, 9 aldehydes, 6 phenols, 6 furans, 3 acids and 3 benzopyrans. More recently, Pino *et al* (2012) investigated an aged,15 year rum from Cuba using gas chromatography – flame ionisation detection (GC-FID), GC-MS and GC-O (Gas chromatography – olfactometry). GC-O enables the panellist to sniff the sample after injection and evaluate the odour. The retention time of the odour is recorded and compared to previous runs on GC-MS and GC-FID. This study identified and quantified a total of 116 volatiles. Alcohols, unsurprisingly, made up the greatest proportion of volatile compounds (82.4%), with 3-methylbutan-1-ol and 2-methylpropan-1-ol being the major alcohols present. The next prevalent compounds were esters with ethyl acetate, ethyl lactate, ethyl decanoate and diethyl succinate present in the greatest concentrations. It was considered that these alcohols and esters were mainly produced during microbial fermentation of the molasses (Pino *et al*; 2012). Pino *et al* (2012) also isolated lactones (3) and phenols (13) which were thought to predominately come from the oak barrels during maturation. While 12 acids were detected, they were considered to have little impact on rum flavour due to their low concentrations and low flavour thresholds. Of these acids, the main ones found were acetic acid, octanoic acid, decanoic acid and hexanoic acid.

Sampiao *et al* (2008) compared the key flavour volatiles (61 analytes) in 44 samples of Cuban and non-Cuban rums. Of these, 8 compounds (isoamyl alcohol, n-propyl alcohol, copper, iron, furfuraldehyde, benzaldehyde, epicatechin and vanillin) were able to comprehensively discriminate between the Cuban and non-Cuban rums. Using statistical methods, the results were clustered into two distinct groups. Further comparison was performed between Cuban rums and those from around the world. Benzaldehyde, isoamyl alcohol, copper and furfuraldehyde had

higher statistical loading values characteristic of Cuban rums when compared to compounds whose statistical loading values were lower and thus coincided with rums from other countries. The analysis also highlighted differences between distillations performed either by pot stills or column stills.

The only previous research conducted on the flavour volatiles of Bundaberg Rum, was performed by Allan (1972) who identified 77 compounds consisting of alcohols, esters, carbonyl compounds, acetals, sulphur compounds and hydrocarbons. This research identified a new compound (1-octen-3-ol) which had not been identified in a rum previously. The importance of methyl-salicylate to rum flavour was also discussed.

Each study mentioned previously identified key flavour compounds, indicative of rum flavour. Appendix A tabulates all compounds found in these studies and includes, where available, the concentrations quantified. A summary table (Table 2.6) highlights the most important sensory components, their detected concentrations and source. A comparison to well known sensory ‘notes’ is included to identify unique flavours or aromas.

Table 2.6 Important flavour volatiles in rum

Compound	ppm	Isolated from	Sensory notes
Esters			
Ethyl acetate[c,f]	73-200	Sugar cane molasses, Jamaican rum	Sweet – pear drops
Ethyl formate[c,f]	10	Sugar cane molasses, Jamaican rum	Characteristic smell of rum
Isoamyl acetate[c,f]	5-10	Sugar cane molasses, Jamaican rum	Banana/pear
Ethyl propionate[c]	50	Jamaican rum	Fruity
Ethyl n-butyrate[c]	220	Jamaican rum	Fruity - pineapple
Ethyl n-valerate[c]	40	Jamaican rum	Pleasant fruity - apple
Ethyl n-hexanoate[c,h,i]	1-40	Jamaican rum, Cuban Rum	Powerful fruity - pineapple
Ethyl n-octanoate[c,h,i,k]	15-50	Jamaican rum, Cuban Rum	Floral fruity - wine
Ethyl n-decanoate[c,h,i,k]	25-130	Jamaican rum, Cuban Rum	Sweet, oily, nut-like, yeasty
Ethyl n-dodecanoate[c,h,i]	12-15	Jamaican rum, Cuban Rum	Floral fruity - waxy
Ethyl n-hexadecanoate[c]	5-50	Jamaican rum	Mild, waxy, sweet
ethyl linoleate[c,f]	50	Jamaican rum	Faintly fruity
Ethyl lactate[c]	10	Jamaican rum	Tart butterscotch, pineapple
Methyl salicylate [c,e]	0.5-25	Jamaican rum, Australian rum	Winter green
Acids			
Acetic acid[c,f]	10.3-35	Sugar cane molasses	Sour vinegar
n- hexanoic acid[c,f]	0.3-15	Jamaican rum	Fruity cheese
n-butyric acid[b,c,f]	0.3-7.5	Sugar cane molasses, high concentrations in Jamaican rum	Creamy sour cheese, fruity
n-valeric acid[c,f]	0.04-7.5	Sugar cane molasses	Earthy cheese
n-propionic acid[b,c,f]	0.2-1.5	Sugar cane molasses	Dairy, fruity
n-octanoic acid[c]	4.3-7.5	Jamaican rum	Rancid vegetable
Alcohols			
1- propanol[c,f,j]	7.5-420	Sugar cane molasses, fermented molasses	Alcoholic ripe fruit
1-butanol[c,f,j]	10	Fermented molasses	Medicinal
Isobutanol[c]	100	Jamaican rum	Fruity wine-like
2-methyl-1-butanol[c,f]	200-210	Sugar cane molasses, fermented molasses	Roasted wine, whisky-like
3-methyl-1-butanol[c,f]	860-1000	Sugar cane molasses, fermented molasses	Fusel, whisky-like
Phenolic compounds			
4-methylguaiacol [c,f]	0.05	Decarboxylation by yeast and bacteria of vanillin	Sweet, coffee, cocoa
Eugenol [a,c,d,f,g,k]	nd-1.36	Extracted from oak during maturation	Spicy, woody clove
Vanillin [c,f,j,k]	0.25	Cane molasses Extracted from oak during maturation Cuban Rum	Vanilla, sweet

[a]Maarse and ten Noever de Brauw (1966),[b]Nykanen (1968), [c]Liebich *et al.* (1970), [d]Timmer *et al* (1971), [e]Allan (1972), [f]Lehtonen and Suomalainen (1977), [g]Lehtonen (1983), [h]Pino *et al.* (2002), [i]Pino *et al* (2007), [j]Sampaio *et al* (2008), [k]Pino *et al* (2012).

2.4 The Role of Microorganisms in Rum Production

The earliest known scientific study into the microbiology of rum was undertaken by Bryan Higgins, an Irish naturalist and philosopher in chemistry (Keattch, 1991). He resided in Jamaica from 1797-1799. It took a further 100 years for Greig (1895) to publish an article entitled “The Jamaica Yeasts”, a paper outlining preliminary studies of a yeast found in association with Jamaican molasses used at a Jamaican rum distillery. Studies on the microbiology of rum production date back to the 1890s with some of the earliest works being reported by Greig (1895), Pairault (1903) Allan (1906), and Ashby (1909). Despite more than 100 years of research since that time, microbiological understanding of the process remains very limited, and is significantly lagging compared with knowledge of the microbiology of other alcoholic beverages such as beer, wine or whisky.

There are two main points in the production chain where microorganisms impact on the process and determine rum quality and process efficiency. These points are storage and preparation of the raw materials (principally molasses, sugar cane syrup or sugar cane juice), and the process of fermentation. Since this thesis concerns molasses style rums, the following sections will focus on the microbiology of molasses and the microbiology of molasses fermentation. Because dunder plays a unique role in the process, a section will also consider its microbiology. A final section will give a brief overview of the microbiology of cachaça style rums.

In developing these sections, particular consideration will be given to:

- Which microbial species occur throughout the production chain
- The survival and growth behaviour of these microbial species throughout production and the various factors that affect this behaviour
- How the biochemistry of this microbial growth changes the chemical composition of molasses and impacts on the flavour and sensory quality of the final rum product.

2.4.1 Microbial Ecology of Molasses

The chemical composition and properties of molasses as they relate to rum production have been described in Section 2.2.1.3. The high concentrations of sugars, low water activity and relatively low pH make these raw materials unfavourable environments for growth and survival of microorganisms. Nevertheless, the literature contains sporadic reports on the isolation of yeasts and bacteria from these materials. The microbiological content of molasses, initially, consists only of endospore forming bacterial species as a result of the high temperatures involved in sugar extraction. An indigenous microflora will establish through contaminations occurring during transportation, storage, contact with processing equipment and general exposure to the elements (Moroz, 1963; Watson, 1993). Molasses is stored in various kinds of containers, depending on manufacturing conditions, from open air wells to secure stainless steel tanks. During this time, microbial contaminants have the potential to grow and produce metabolic end products that may impact on rum quality. This indigenous microflora, itself, is diverse and may impact on the ecology of the fermentation process.

While there are isolated reports on the recovery of microorganisms from molasses, there have not been any systematic investigations of the microbiology of molasses or syrups during storage for rum production. Table 2.7 summarises literature detailing microorganisms present in molasses (and other related products such as sugar cane and cane juice). Sugar cane and sugar cane juice have been included as there are limited previous studies detailing the microorganisms naturally present in molasses.

Table 2.7 Microorganisms found in raw materials, such as molasses and sugar cane juice, associated with alcoholic fermentations for rum production.

Reference	Raw material	Species isolated	
Hall *et al* (1935)	Molasses	*Zygosaccharomyces nussbaumeri,* *Zygosaccharomyces major,*	*Penicillium,* *Mucor,* ***Clostridium saccharolyticum***
Owen (1949)	Molasses	*Zygosaccharomyces nussbaumeri* *Zygosaccharomyces major*	*Zygosaccharomyces globiformis* *Schizosaccharomyces pombe*
El-Tabey Shehata (1960)	Sugar cane juice	*Candida guilliermondii* *Candida intermedia* var. ethanophila *Candida mycoderma* *Candida tropicalis* *Endomyces magnusii* *Kloeckera apiculata* *Pichia fermentans* *Pichia membranaefaciens* *Saccharomyces acidifaciens*	*Saccharomyces cerevisiae* *Saccharomyces carlsbergensis* var. alcoholophila *Saccharomyces marxianus* *Saccharomyces microellipsodes* *Saccharomyces rosei* *Saccharomycodes ludwigii* *Torulopsis glabrata* *Torulopsis stellata* *Torulopsis stellata* var. cambresieri
Kampen (1975)	Molasses	*Saccharomyces cerevisiae*	
Parfait and Sabin (1975)	Molasses/ cane juice	*Candida krusei* *Candida pseudotropicalis* *Candida tropicalis* *Hansenula anomala* *Hansenula minuta* *Saccharomyces aceti* *Saccharomyces acidifaciens*	*Saccharomyces cerevisiae* *Saccharomyces chevalieri* *Saccharomyces rouxii* *Torulopsis candida* *Torulopsis glabrata* *Torulopsis globosa* *Torulopsis stellata*
Tilbury (1980)	Molasses/ raw cane sugar	*Saccharomyces heterogenicus*	
Ganou-Parfait *et al* (1989)	Molasses	***Propionibacterium jensenii*** ***Lactobacillus fructivorans***	***Leuconostoc paramesenteroides***
Bonilla-Salinas *et al.* (1995)	Molasses	*Schizosaccharomyces pombe* *Saccharomyces cerevisiae*	*Torulaspora delbrueckii* *Cryptococcus albidus* var. albidus
Todorov & Dicks (2005)	Molasses	***Lactobacillus plantarum***	

Note: Bacterial species given in the table are shown in **bold** font

There is a noticeable difference between fresh “green” molasses and “aged” molasses. It has been debated that the molasses itself undergoes spontaneous chemical alteration which leads to this “ageing” (Owen, 1911). Because of the heat processes involved, freshly produced molasses, or syrup, contain few microorganisms (Owen, 1911; Browne, 1929). Browne (1929) analysed molasses samples over a 14 year period and failed to detect the presence of any yeasts, moulds or bacteria.

More recently, several research groups have revealed that molasses harbours complex microflora, including yeast and bacteria (Hall *et al*., 1935; Owen, 1949; Bonilla-Salinas *et al.*, 1995; Fahrasmane & Ganou-Parfait, 1998)

Hall *et al.* (1935) examined the microflora of Barbados molasses. Using microscopy, they observed various yeast and bacterial cells but were unsuccessful in obtaining culturable colonies of yeast when directly plated on to malt extract agar or cane sirup [*sic*] agar (an agar made by 1:2 of cane sirup [*sic*] to nutrient agar pH to 6.8-7.0). Hall *et al.* (1935) improved their isolation technique by the addition of an enrichment step prior to plating. This extra step allowed for 11 cultures of two yeasts (*Zygosaccharomyces nussbaumeri* and *Zygosaccharomyces major*) to be obtained from two samples of molasses (1932 and 1933). A study was concurrently run to isolate the bacteria present in Barbados molasses. *Clostridium saccharolyticum* was consistently isolated and it was concluded that it was an important organism in the development of flavour volatiles in rum fermentations (Hall *et al*, 1935). Mould was frequently isolated after incubation at a variety of temperatures and on a variety of media. The most predominant moulds were considered to be in the genera *Penicillium* and *Mucor*. Species of these moulds were not determined.

Owen (1949) discussed four origins of microorganisms in relation to their presence in molasses. These included the “epiphytic” flora of the sugar cane plant itself, microorganisms endemic to the soil that the plants grow in, microflora introduced to the mills and refineries via contamination through air particulates, and microorganisms introduced through a secondary raw material used to extract sugar from molasses and sugar cane juice (for example, starch used in production of confectionary sugars). While Owen did not perform any experiments at this time, his theories were informative and provided a “check list” of potential sources of microflora present in distilleries. To date, no one has done a complete survey, including all of these origin points, of a distillery.

El-Tabey Shehata (1960) undertook research into the natural yeast microflora of sugarcane and the associated juice (both fresh and fermented) at sugar factories in Brazil. Forty three yeast cultures were recovered from the 14 samples taken from five different factories. Isolated from sugar cane plants and fresh juice, species of *Saccharomyces, Candida, Pichia* and *Torulopsis* were the most predominant. Yeasts from only three genera were isolated from fermenting juice (*Saccharomyces, Candida* and *Schizosaccharomyces*), while the most frequently isolated yeasts from fresh juice were *S. cerevisiae* and *Candida krusei.*

From the 1970's to the mid 1990's several research groups investigated both yeast and bacterial species in molasses, sugar cane and sugar cane juice. Parfait and Sabin (1975) examined the prevalence of yeast species in both molasses and cane juice. Fourteen species were isolated and identified (Table 2.7). As part of a larger study (discussed further in Section 2.4.2), Ganou-Parfait *et al* (1989) isolated three bacteria (*Propionibacterium jensenii*, *Lactobacillus fructivorans* and *Leuconostoc paramesenteroides)* from molasses.

Bonilla-Salinas *et al* (1995) isolated and identified 13 yeast strains from sugar cane molasses produced in Mexico. The most frequently isolated yeast was *Schizosaccharomyces pombe* (isolated 7 times). This was the first study to identify killer strains in molasses, including *Schizosaccharomyces pombe* which had not previously been known to have killer strains. This research may help further work into starter cultures for the rum industry as discussed later in Section 2.4.4.

Previous research has concentrated on isolation and identification of both yeast and bacterial species. There has been limited research in quantifying population levels of microorganisms in molasses when used for rum production. Bacteria are generally thought to exist in populations between 10^2-10^3 bacteria/g (Fahrasmane and Ganou-Parfait, 1998) while, to date, there is no published population data for yeast species isolated from molasses.

2.4.2 Microbial Ecology of Molasses Fermentation for Rum Production

Molasses has a high sugar content and a relatively low pH which naturally selects for the growth of yeasts. Consequently, yeasts are the most prevalent microorganisms of molasses based rum fermentations, with strains of *Saccharomyces cerevisiae* being the most frequently isolated (Lehtonen & Suomalainen, 1977; Nicol, 2003). Nevertheless, various bacterial species have been associated with these fermentations and need to be considered as part of the microbial ecology. Table 2.8 lists various studies that have reported the isolation of yeasts and bacteria from molasses based rum fermentations and sugar cane juice rhum agricole fermentations.

Table 2.8 Microbial species associated with the fermentation of molasses or sugar cane juice for rum or *rum agricole* production

Reference	Fermentation medium	Species isolated	
Greig (1895)	Molasses	*Schizozaccharomyces mellacei*	
Kampen (1975)	Molasses	***Lactobacillus ssp***	
Parfait & Sabin (1975)	Molasses	*Schizosaccharomyces pombe* *Schizosaccharomyces ssp*	Saccharomyces cerevisiae
Ganou-Parfait *et al*. (1987)	Molasses	***Bacillus ateriimus*** ***Bacillus cereus*** ***Bacillus megatherium***	***Bacillus mesentericus*** ***Bacillus subtilis***
Fahrasmane *et al* (1988)	Molasses	*Schizosaccharomyes pombe* *Schizosaccharomyces japonicus* ***Clostridium* spp.**	*Schizosaccharomyces malidevorans* *Saccharomyces cerevisiae* ***Bacillus* spp.**
Ganou-Parfait *et al.* (1989)	Molasses/ sugar cane/Soils/Waters	***Micrococcus luteus*** ***Micrococcus varians*** ***Bacillus cereus*** ***Bacillus subtilis*** ***Bacillus megaterium*** ***Bacillus sphaericus*** ***Brevibacterium incertae sedis*** ***Corynebacterium incertae sedis*** ***Erysipelothrix*** ***Kurthia zopfli*** ***Listeria*** ***Microbacterium lacticum*** ***Propionibacterium acidipropionici*** ***Propionibacterium jensenii*** ***Propionibacterium freeudenreichii***	***Lactobacillus fermentum*** ***Lactobacillus fructivorans*** ***Lactobacillus hilgardii*** ***Lactobacillus viridescens*** ***Leuconostoc mesenteroides*** ***Leuconostoc paramesenteroides*** ***Clostridium butyricum*** ***Clostridium beijerinckii*** ***Clostridium acetobutylicum*** ***Clostridium felsineum*** ***Clostridium puniceum*** ***Clostridium thermosulfurigenes*** ***Clostridium thermohydrosulfuricum*** ***Clostridium sporogenes*** ***Clostridium bifermentans***
Fahrasmane & Ganou-Parfait (1998)	Molasses and sugar cane juice	*Saccharomyces cerevisiae* *Saccharomyces chevalieri* *Saccharomyces rouxii* *Saccharomyces aceti* *Saccharomyces microellipsodes* *Saccharomyces delbrueckii*	*Saccharomyces carlsbergensis* *Schizosaccharomyces pombe* *Hansenula anomala* *Torulopsis glabrata* *Torulopsis stellata*

Note: Bacterial species given in the table are shown in **bold** font

2.4.2.1 Yeasts

Yeasts are essential to fermentation of molasses for rum production. Greig's (1895) article "The Jamaica Yeast" described a yeast found in Jamaican molasses

used in rum production. This yeast was later described by Jorgensen and Holm as *Schizosaccharomyces mellacei* (Jorgensen, 1909). Early studies noted two broad groups of yeasts associated with rum fermentations, notably, the more prevalent *Saccharomyces* yeasts where cell division occurred by budding, and the *Schizosaccharomyces* yeasts where cell division occurred by fission. The two types could be readily distinguished by microscopic examination (Kayser, 1913).

Arroyo (1945) discussed at length the impact of fission yeasts versus budding yeasts on rum production, but precise details about the species involved and their growth kinetics were rarely mentioned. Heavy style rums, namely, those more traditional in production and flavour would require fermentation with a predominance of *Schizosaccharomyces* or fission yeast and minor contributions from budding yeasts. Such fermentations were generally slower, and produced rums with higher contents of esters, higher alcohols, organic acids and aldehydes which combine to contribute stronger organoleptic tastes and aromas. For production of lighter rums, Arroyo (1945a) postulated a faster fermentation using, budding *Saccharomyces* yeasts. This type of yeast would produce fewer congeners such as organic acids, aldehydes, esters etc., thus giving a rum with a “cleaner” taste. These conclusions were supported by later studies of Parfait and Sabin (1975) and Fahrasmane *et al.* (1985).

Parfait and Sabin (1975), while investigating fermentation in the French West Indies, reported that *Schizosaccharomyces* spp. were predominant in fermentation media used for heavy flavour rum production, while *Saccharomyces* spp. were predominant in wild fermentation and seeded media. The two different species of yeast exerted different effects on flavour development during fermentation. Fission type yeasts such as *Schizosaccharomyces pombe* produced heavy flavoured rums, while faster fermenting budding type yeasts such as *Saccharomyces cerevisiae* produced light flavoured rums.

Fahrasmane *et al.* (1985) found that *Saccharomyces cerevisiae* produced greater

concentrations of higher alcohols and short chain fatty acids than *Schizosaccharomyces* strains. *S. cerevisiae* also fermented the molasses medium more rapidly than *Schizosaccharomyces* strains.

2.4.2.2 Bacteria

Bacteria can impact favourably and detrimentally to the production of rum. Greig (1893) was the first to document the presence of bacteria in rum fermentations. While he did not conduct specific studies on these bacteria, he suggested that their growth be suppressed by the inoculation of yeast cultures. The possibility of a positive role of bacteria in rum fermentations was first suggested by Allan (1906). While investigating Jamaican rum fermentations, Allan (1906) highlighted the presence of two *Bacillus* species. He suggested that such bacteria might utilise dead yeast cells as nutrients at the end of yeast fermentation, and that organic acid and higher alcohol production by these bacteria would contribute the characteristic rum flavour.

Several authors (Kampen, 1974; Ganou-Parfait *et al*, 1987 & 1989; Fahrasmane, 1988) have reported the qualitative presence of bacterial species in molasses rum fermentations but more detailed information about populations and frequency of occurrence was scant and inconsistent. Bacterial populations, when reported, were generally low (10^2-10^3 CFU/mL) and reflected a diversity of species within the genera *Bacillus, Brevibacterium, Clostridium, Corynebacterium, Lactobacillus* and *Propionibacterium* (Ganou-Parfait *et al,* 1987 & 1989; Fahrasmane *et al,* 1988).

Clostridium saccharobutyrium (isolated from sugar cane bagasse) has been shown to have beneficial effects by increasing the rate of formation of alcohol during yeast fermentation of molasses (Fahrasmane *et al*, 1988). Also, these workers proposed that *Clostridium* species found in rum distilleries could contribute to the production of volatile acids such as acetic, butyric, caproic, heptanic and propionic acids. These acids are important precursors for ester formation that is essential for rum

aroma and flavour.

Ganou-Parfait *et al* (1989) investigated the bacterial species associated with rum production. This research comes the closest to a complete survey based on the four potential origins of microflora postulated by Owen (1949). The study examined the source of the bacteria (cane, molasses, water, and soils), their optimum growth temperatures, optimum pH, optimal growth period during fermentation, metabolic products and effect (either positive or negative) on the yield of rum and its organoleptic qualities. It was suggested that the development of heavy rum aromas relies significantly on the presence and activity of bacterial species. The presence of propionic acid bacteria, such as *Propionibacterium jensenii,* is associated with the production of propionic acid. High levels of this acid differentiate rum from other distilled alcoholic beverages (Ganou-Parfait *et al*, 1989). High populations of propionic acid bacteria can, however, be detrimental to flavour due to the production of highly acidified rum (Fahrasmane & Ganou-Parfait, 1998). Lactic acid bacteria, such as *Lactobacillus* species, produce metabolites which can acidify the medium and develop more complex organoleptic properties such as aldehydes, esters and diacetyl (Jay, 1982). Some lactic acid bacteria can, however, be detrimental to finished rum by producing dextrins and other polysaccharides during sugar processing and molasses storage. These bacteria utilise sugars (e.g. metabolise sucrose to polysaccharides) that otherwise would have been fermented to produce ethanol and other flavour volatiles.

From the limited research conducted to date, it is evident that bacteria are part of the microbial ecology of rum fermentation. Their potential impacts can be summarized as: positive and unique contributions to rum flavour; detrimental effects on rum flavour; and reduction in process efficiency by modulating yeast growth and their production of ethanol. The extent to which they will contribute positively or negatively to the process will be determined by the species that are present and their ability to compete with the growth of yeasts. More research is needed to better define and understand the role of bacteria in rum fermentations

and will be a focus of this thesis.

2.4.3 Microbiology of Dunder

The use of dunder in rum production has been described in Section 2.2.1.5. This section considers its microbiology.

Most definitions accept dunder as the material remaining in the still after distillation of the fermented molasses (Section 2.2.1.5). Yeast and bacterial cells associated with the fermentation will be dead as they would have been inactivated by the heat (>80°C) during the distillation process. Consequently, dunder originating directly from the still should be sterile (Kampen, 1975). If it is subsequently stored, it is likely to become contaminated and support microbial growth, the extent of which will depend on the conditions of storage such as time, temperature and hygiene of the environment. There are several reports (Wustenfeld & Haeseler, 1953; l'Anson, 1971; Murtagh, 1995b) that refer to the contamination and growth of microorganisms in stored dunder, but no scientific evidence has been presented to support these claims or to provide data about the species and populations of microorganisms that might be present. In some cases, such growth in dunder is encouraged to serve as a source of unique microflora for inoculation into the molasses fermentation (Wustenfeld & Haeseler, 1953; l'Anson, 1971; Murtagh, 199b, Nicol, 2003)

Given the wide spread use and perceived importance of dunder in rum production, more research is needed about its microbiological status and will be examined in Chapter 5 of this thesis.

2.4.4 Use of Starter Cultures in Molasses Fermentation for Rum Production

As mentioned in Section 2.1.1, rum fermentation is a spontaneous process

conducted by indigenous yeasts and bacteria originating from uncontrolled “wild” fermentations. The concept of using starter cultures for rum fermentations has been considered since the late 1890s. Arroyo (1945a) gives an historical account of the early studies concerning the use of starter cultures in rum production.

The notion of using starter cultures to better control alcoholic fermentations is well established in the brewing and wine industries (Fleet, 1998; Mateo *et al*, 2001). The ability to control such fermentations meant that greater batch to batch similarity was achieved, giving products which remained consistent in flavour and aroma. This led to greater consumer loyalty to particular brands and, consequently, increased profits. The rum industry, with the exception of small artisanal distilleries, has followed other fermented beverages towards more commercially driven production capabilities. The production of clearer, lighter rums came as a direct result of the starter culture era. Nevertheless, the philosophy to rum production became divided over the use of yeast starter cultures.

Pairault (1903) was the first to suggest that starter culture yeasts for rum production should be selected on the basis that they are well adapted to growth and fermentation in molasses. This concept was further supported by Kayser (1913), who was behind the push for pure culture fermentations containing only the selected yeasts. Both Pairault (1903) and Kayser (1913) recognized that bacteria were also endemic to many rum processes but were of the view that they impacted negatively on production efficiency and quality. Consequently, use of yeast starter cultures would overcome these issues. Prompted by these researchers, various distilleries isolated and identified the main yeast strains responsible for their fermentations, and these included strains of *S.cerevisiae* and *Schiz. pombe*. The prevalence of either (or both) of these species of yeast in fermentations saw many distilleries adopt them as starter cultures (Arroyo, 1945a).

During the 1970s, rum distilleries commenced using dried baker’s yeast as a cheap, readily available and easily stored alternative to laboratory maintained starter cultures. Fermentation efficiency was boosted and a more consistent

product was able to be produced. The use of dunder was no longer required for these types of fermentations due to baker's yeasts sensitivity to the high acidity provided by dunder. The advent of lighter, clear rums was, thereby, initiated (Fahrasmane & Ganou-Parfait, 1998).

While many considered yeasts to be the main agent of rum fermentation and the main target for use as starter cultures (Arroyo, 1945a), there were other researchers who considered that bacteria were also important to the fermentation. Allan (1906) and Ashby (1909) studied Jamaican rums and advocated the importance of bacteria in wild type fermentations for the development of the complex rum aroma found in heavy bodied rums of the region.

Rocques (1927) showed that, while the alcohol yield and efficiency of production increased with the use of yeast starter cultures, the finished rum was low in acid and ester concentrations, high in higher alcohols, and consequently lacked the characteristic flavour and aroma attributed to rum. Such issues caused many distilleries to revert to older practices of wild, uncontrolled fermentations.

To accommodate the concern about bacterial contributions to rum fermentation, Arroyo (1942, 1945a, b) suggested three possible approaches to the use of yeast starter cultures. These were; use with a sterilised molasses medium in aseptic fermenters (a completely controlled environment); use with a partially sterilised fermentation medium; or use with a non-sterilised fermentation medium. The aseptic fermenter would give results such as those obtained by Rocques (1927), containing ethanol but fewer distinguishing "rum-like" aromas. Partial sterilisation of fermentation media may lead to the growth of any bacterial species present, depending on the length of fermentation. The final scenario, the non-sterilised fermentation media, would rely on the selected starter culture out-competing the natural microflora already present and should maximize the growth of the bacterial species found in rum fermentations and their contribution to more complex characteristics of rum flavour (Arroyo, 1945c).

Table 2.9 outlines desirable traits that rum distillery yeasts should possess for maximum efficiency during fermentation, and these have been used to develop specific starter cultures for rum production. Several companies sell distillers yeasts for rum production and these are mentioned in Section 2.2.2.1.

Table 2.9 Properties required by rum yeasts to be commercially successful as a starter culture

Properties
• Conduct strong rapid alcoholic fermentation of molasses (grow at appropriate pH , Aw , temperature, give high ethanol production) • Initiate rapid growth with minimal lag phase so as to outcompete indigenous yeasts and bacteria • Ferment molasses to give desirable sensory metabolites consistent with good rum characteristics • Able to grow under large scale propagation protocols • Be tolerable to preservation by freezing and lyophilisation • Must not be pathogenic • Must not form toxins or antibiotics

2.4.5 Microbiology of Other Distilled Beverages; Cachaça and Whisky

As mentioned in the previous sections, progress in understanding the microbiology and biochemistry of rum fermentation has been very limited despite some 100 years of research. In contrast, there has been significant recent research into the microbiology of cachaça production and whisky production, the developments of which may guide future research on rum microbiology. These developments are summarized in this section.

2.4.5.1 Microbiology of Cachaça Fermentation

As mentioned previously (Section 2.2.5), cachaça is a rum-like, distilled alcoholic beverage produced from fermented sugar cane juice and is produced mainly in Brazil. Sugar cane is crushed to extract the juice which is then immediately fermented. Fermentation may occur as a spontaneous process from the growth of

indigenous yeasts that occur as natural contaminants, or it may be conducted by inoculation with a traditional starter preparation, or selected strains of yeasts, usually, *S. cerevisiae*. Fermentation usually takes about 24-48 h after which the fermented material is distilled to give the cachaça product. Details of the process are discussed by Faria *et al* (2003).

Research into the microbiology of cachaça fermentations has been steadily growing in depth and popularity since the 1990s, especially among Brazilian researchers. Whether conducted by traditional or inoculated processes, the fermentation is dominated by the growth of *S. cerevisiae* and this species is considered to be the key driver of the fermentation. Usually, this species grows to maximum populations of 10^8-10^9 CFU/ml within 36-48 h, after which the fermentation is terminated. Some key studies on the yeast ecology of the fermentation are those of El-Tabey Shehata (1959), Morais *et al*. (1997), Schwan *et al* (2001), Pataro *et al* (2000), de Araujo Vicente *et al.* (2006) and Duarte *et al* (2013). These studies also report the contribution of other yeasts to the fermentation. Such species are, usually, from the genera *Candida, Debaryomyces, Schizosaccharomyces, Kluyveromyces, Pichia* and *Hanseniaspora* but were isolated less frequently than *S. cerevisiae*. They were mostly found during the early stages of cachaça fermentations after which they died off, giving way to the dominance of *S. cerevisiae*

Phenotypic and molecular analyses of isolates of *S. cerevisiae* from cachaça fermentations show substantial diversity within the strains examined, suggesting adaptation to particular process conditions and geographical locations (Badotti *et al* 2010, 2013). This variation can account for the different flavour profiles of cachacas obtained from the different distilleries (Badotti *et al* 2010) and has led to the selection of particular strains for development as unique starter cultures in recent years (Oliveira *et al*, 2004; Gomes *et al*, 2007; Marini *et al*, 2009, Campos *et al*, 2010).

Although yeasts are primarily responsible for cachaça fermentations, Schwan *et al*

(2001) also noted the presence of bacteria, especially lactic acid bacteria, throughout these fermentations. Subsequent studies by Duarte *et al* (2011) investigated cachaça fermentations inoculated with a mixture of *S cerevisiae* and *Lactobacillus fermentum* and demonstrated how the flavour profile of cachaça could be modulated by the growth of bacteria. Consequently, further research is needed to better understand the potential role of bacteria in cachaça fermentations.

2.4.5.2 Microbiology of Whisky Fermentation

Whisky is produced by fermenting the liquid extract obtained by mashing of malted barley. This liquid contains the sugars and other nutrients that are metabolised by microorganisms to produce ethanol and other aromatic compounds. The fermented extract is distilled, matured and blended to give the final product. Details of the process have been reviewed by Piggott and Conner (2003) and Walker (2012). Microbial fermentation of the wort or malt extract is a key operation in the production chain, and the microbiology of this step has been discussed by Suomalainen (1971) Priest and Pleasants (1988), Barbour and Priest (1988) Fleet (1998), van Beek and Priest (2002) and Collicutt (2009 a, b).

Early studies revealed the predominance and importance of yeasts in the fermentation and key species identified were isolates of *Saccharomyces cerevisiae* (Sharp & Watson, 1979). Subsequently, isolates of this yeast were selected, purified and developed as strains of distiller's yeasts that became commercially available for use as starter cultures to guide and better control the fermentation (Jones, 1998). The predominant strains have been commercially marketed by Kerry Ingredients and Flavours as "M" and "MX" strains, by Mauri Products as "Pinnacle" yeast, and by Lallemand Ethanol Technology as DistillaMax©. The cultures of Kerry Ingredients and Flavours and Mauri Products are used in more traditional processes to give a fuller bodied product, while Lallemand's, DistillaMax© is marketed for the grain whisky or neutral spirit fermentations

(Walker, 2012; Russel & Stewart, 2014).

In recent years, lactic acid bacteria have been found to have a significant role in whisky fermentation and, depending on how the process is managed, they can grow to populations of 10^6-10^8 CFU/ml after 48-72 h of fermentation. The main species isolated from such fermentations are *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis, Weissella confuse* and *Lactobacillus paracasei*. The potential impacts of these bacteria on the fermentation have been considered as:(i) a decrease in ethanol production by the yeasts due to sugar consumption by the bacteria;(ii) an enhancement of whisky flavours due to production of higher alcohols and other congeners; and (iii) a detrimental effect if their growth is unregulated, leading to stuck alcoholic fermentation and the production of “off” flavours (Simpson *et al,* 2001; van Beek & Priest, 2003; Cachat & Priest, 2005; van Beek & Priest, 2000, 2001 & 2003).

Although cachaça and whisky fermentations were once thought to be predominantly processes attributed to yeasts, principally strains of *S. cerevisiae*, there is increasing evidence that bacteria, especially lactic acid bacteria, may positively contribute to these fermentations. Although cachaça, whisky and rum production are based on different raw materials, the microbial ecology of the processes has many similarities. On this basis, it is likely that bacteria, especially lactic acid bacteria, may play a more significant role in rum production than previously thought.

2.5 SUMMARY

A systematic investigation into the microbial species which impact on the processes involved in the manufacture of rum will be undertaken. Rum is produced by microbial fermentation of molasses, a waste product generated by the cane sugar industry. The rate and extent of this fermentation determines process efficiency. Several yeast and bacterial species contribute to rum fermentation but the related ecology is poorly defined and understood. Through a combination of ecological studies, controlled fermentations and distillations, and chemical analysis, the impact of particular microbial species on rum flavour will be determined.

CHAPTER 3.

THE MICROBIAL ECOLOGY OF A PROCESS FOR RUM PRODUCTION

3.1 INTRODUCTION

Rum is a distilled alcoholic beverage obtained from the fermentation of sugar cane molasses or sugar cane juice (Arroyo, 1945a; Clutton, 1974; Kampen, 1975; Lehtonen & Suomalainen, 1977; Bluhm, 1983 and Nicol, 2003). In contrast to many other alcoholic beverages such as beer (Iserentant, 1995), wine (Fleet, 1998; Mateo *et al*, 2001) and distilled products such as whisky (Suomalainen, 1971; Priest & Pleasants, 1988; Barbour & Priest, 1988; Fleet, 1998; van Beek & Priest, 2002 and Collicutt, 2009b), the microbial ecology of the process has not been extensively studied. The little information known about the microbiology of rum fermentation has been summarised in reviews by Lehtonen & Suomalainen (1977) and Nicol (2003). It is concluded that the yeast, *S. cerevisiae*, is the key organism that conducts the alcoholic fermentation of molasses and it has been developed as a starter culture for this purpose but, in some processes, indigenous strains of *Schizosaccharomyces pombe* may also make a contribution (Hall, 1935; Parfait & Sabin, 1975; Ganou-Parfait *et al*, 1989; Bonilla-Salinas *et al,* 1995; Todorov & Dicks, 2004). A few studies have reported the presence and significance of bacteria during rum fermentations but details about their frequency of occurrence and growth during such fermentations have not been investigated (Kampen, 1974; Ganou-Parfait *et al,* 1987 & 1989; Fahrasmane *et al*, 1988). Such species include *Clostridium saccharobutyricum* (Fahrasmane *et al*, 1988), *Propionibacterium jensenii* (Ganou-Parfait *et al,* 1989) and lactic acid bacteria (Kampen, 1975;

Ganou-Parfait *et al,* 1989). It has been suggested that these bacteria may contribute positively or negatively to the quality of the final product, but definitive conclusions about their contributions require more detailed investigation. Bacterial populations, when reported, were generally low (10^2 – 10^3 CFU/mL).

To provide a greater knowledge about the microbiology of the rum fermentation process, this Chapter aims to systematically study the microbial ecology of a rum distillery located at Bundaberg, Queensland, Australia; from raw materials (molasses, dunder, yeast and water) through propagation and fermentation.

3.2 MATERIALS AND METHODS

3.2.1 Process Outline and Sample Selection

The process of rum production at the Bundaberg distillery, Queensland, was investigated. This process is outlined in Figure 3.1 and is described in detail in Section 3.3.1 of the Results section.

Samples for microbiological examination were taken at the sites listed in Table 3.1 and shown in Figure 3.1. They were taken during commercial operation of the facility over a four year period (March 2006 to April 2010). Samples (50 mL) were taken in duplicate under aseptic conditions and stored at 4°C until microbiological analysis, within 24 h. Some sites had fixed sampling ports that were sterilized by flushing with 70% ethanol, after which sample was then flushed through the port in order to obtain a representative fraction. Some samples were collected using 10 mL syringe - vaccutainers (Becton Dickinson). In these cases, samples from three vaccutainers were transferred into sterile plastic containers (Sarstedt) and mixed to produce a representative 30 mL sample.

Table 3.1 Sampling sites for microbiological study of a rum distillery

Stage/Sampling point
Molasses preparation
Molasses supply tank
Dunder
Dunder and mud line
Dilution tank
Clarifier
Surge tank (50° Brix)
Surge tank (30° Brix)
Floc addition
Town water
Yeast propagation in molasses medium
Yeast propagation Vessel A (early)
Yeast propagation Vessel A (late)
Yeast propagation Vessel B before transfer to Yeast vessel C
Yeast propagation Vessel C before transfer to Yeast vessel D
Yeast vessel D prior to transfer to Fermenter
Molasses fermentation
Fermenter sample post transfer of yeast (0 h)
Fermenter sample (6 h)
Fermenter sample (12 h)
Fermenter sample (18 h)
Fermenter sample (24 h)
Fermenter sample (36 h)
Fermented molasses in buffer tank A
Fermented molasses in buffer tank B

3.2.2 Isolation, Enumeration and Identification of Microorganisms

The microbial flora of samples was examined by (1) culture plating on agar media; (2) enrichment culture followed by plating on agar media

3.2.2.1 Yeasts

The samples were serially diluted in 0.1 % Bacteriological Peptone Water, and 0.1 mL spread inoculated, in duplicate, onto plates of Malt Extract Agar (MEA) (Oxoid) and Wallerstein Differential Nutrient Agar (WL) (Oxoid) supplemented to contain 100 µg/mL of oxytetracycline (Sigma) to restrict bacterial growth. Plates were incubated at 25°C for 48 h, after which time colonies were counted. Predominant colony morphologies were noted and approximately five representative isolates of each type were purified by streaking onto plates of MEA. Stock cultures were stored at -80°C under 30 % glycerol until used for identification.

To determine the presence of low populations of yeasts in some samples, subsamples (1 mL) were subject to enrichment culture in 50 mL of Malt Extract (ME) broth (Oxoid) for 24-48 h at 25°C. The cultures were then tested for the presence of viable yeasts by streak plating samples onto MEA.

Extraction and sequencing of ribosomal DNA

DNA was extracted from disrupted cells by a modification of the method described by Kowalchuk *et al* (1997). Yeast isolates were grown overnight in ME broth. Samples (1-2 mL) of this culture were centrifuged (2 min at 10,000 g) at room temperature in a Beckman Microfuge 18 Centrifuge (Beckman Coulter Inc., Fullerton, CA, USA) to obtain a cell pellet. The cell pellet, contained within a 2 mL screw capped microtube, was resuspended in 0.2 mL of extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA [pH 8], 100 mM NaCl, 1% sodium dodecyl sulphate [SDS]), to which was added 0.3 g zirconia/silica beads (diameter 0.5 mm; Daintree Scientific, Tasmania, Australia), and 0.2 mL of phenol:chloroform:isoamyl alcohol (24:24:1 (v:v:v) Sigma-Aldrich, Australia). The samples were shaken at 500 rpm for 1 min in a mini-bead beater (Biospec Products, OK, USA). After centrifugation for 10 min (15,000 g at 4°C), 0.4 mL of the upper layer was removed. DNA was precipitated by addition of 0.4 mL of isopropanol and the mix allowed to stand for 24 h at -20°C. After centrifugation for 10 min (15,000 g), the pellet was washed once with 70% ethanol and allowed to air dry. The dried pellet was dissolved in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) and stored at -20°C until use in PCR.

Extracted DNA was used as template DNA in the polymerase chain reaction (PCR) to amplify sections of the 26S ribosomal RNA region. The extracted DNA was amplified by PCR using universal primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') for identification by partial 26S rDNA sequence analysis (Kurtzman and Robnett, 1998). These primers were obtained from Sigma Genosys, NSW, Australia.

The PCR reaction mixtures contained 10 mmol l^{-1} Tris HCl (pH 8.3), 50 mmol l^{-1} KCl, 0.2 µmol l^{-1} of each primer, 200 µmol l^{-1} of each dNTP (Roche Diagnostics, Indianapolis, IN, USA), 1.0 mmol l^{-1} $MgCl_2$, 1.25 U of Gold *Taq* DNA Polymerase (AmpliTaq™, Roche Molecular Systems, Branchburg, NJ, USA) and 10 ng of purified template DNA in 50 µl final volume. Amplification was performed under the following programme: initial denaturation at 95°C for 7 min, 36 cycles at 95°C for 1 min (denaturation), 52°C for 2 min (annealing) and 72°C for 2 min (extension), with the final extension conducted for 10 min at 72°C. Confirmation of amplicons was done by agarose gel electrophoresis after which they were used for sequencing with the ABI PRISM® BigDye™ Terminators v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The products were sequenced at the Ramaciotti Centre for Gene Function Analysis, UNSW, Australia. The resulting sequences underwent DNA similarity searches with the NCBI Blast program using sequences retrieved from the Genebank Database (Karlin & Altschul, 1990).

3.2.2.2 Bacteria

Total bacteria and lactic acid bacteria

The samples were serially diluted in 0.1 % Bacteriological Peptone Water, and 0.1 mL spread inoculated, in duplicate, onto plates of de Man, Rogosa and Sharpe agar (MRS) (Oxoid), Wallerstein Differential Nutrient Agar with Supplement (WLS) (Oxoid), Plate Count Agar (PCA) (Oxoid) and Raka Ray (Oxoid) agar, each supplemented to contain 10 µg/mL of cycloheximide (Sigma) to restrict yeast growth. Plates were incubated either microaerophillically and anaerobically at 30°C for 48 h, after which time colonies were counted. Predominant colony morphologies were noted and five representative isolates of each type were purified by streaking onto plates of MRS or WLS agar. Stock cultures were stored at -80°C under 30% glycerol until used for identification.

To determine the presence of low populations of bacteria in some samples, subsamples (1 mL) were subject to enrichment culture in 50 mL of MRS broth

(Oxoid) for 24-48 h at 30°C. The cultures were then tested for the presence of viable bacteria by streak plating samples onto MRS agar.

Zymomonas* and *Propionibacterium

The samples were serially diluted in 0.1 % Bacteriological Peptone Water, and 0.1 mL spread inoculated, in duplicate, onto plates of Sodium Lactate Agar (SLA) and Universal Beer agar (modified for rum fermentations), each supplemented to contain 10 µg/mL of cycloheximide (Sigma) to restrict yeast growth. SLA was made according to Atlas (2006) with the composition per litre consisting of: agar 15.0 g, Pancreatic digest of casein 10.0 g, sodium lactate 10.0 g, yeast extract 10.0 g and K_2HPO_4 0.25 g. Plates were incubated at 30°C under anaerobic conditions for 5-7 days. Universal Beer agar (Oxoid) was prepared according to directions provided by Oxoid with the addition of 250mL/L of either dunder or fermentation medium to batches. Plates were incubated anaerobically for 3-7 days. Anaerobic conditions were obtained using AnaeroGen© (Oxoid) in appropriate sealed containers.

Clostridium

The samples were serially diluted in 0.1% Bacteriological Peptone Water, and 1 mL was used to prepare duplicate pour plates of Differential Reinforced Clostridial Agar (RCA) (Difco). The plates were incubated anaerobically at 30°C for 2-10 days (plates were checked for growth every 48 h). Anaerobic conditions were obtained using AnaeroGen© (Oxoid) in appropriate sealed containers.

Identification of bacterial isolates.

Bacteria were identified by a combination of phenotypic methods and sequencing of the 16 S ribosomal DNA.

Phenotypic tests

Phenotypic characterization included microscopic examination for cell morphology, Gram staining, and tests for oxidase. Isolates were then selected for identification using API CHL50 test strips (Biomerieux, Durham NC). Cultures were grown at 30°C for 24 h on MRS agar prior to collection and suspension of cell biomass in

sterilised distilled water according to kit instructions. API kits were inoculated with the biomass and incubated at 30°C for 24 h and observed for reactions. Kits were incubated for a further 24 h, reactions recorded and data processed using APIweb™ (http://apiweb.biomerieux.com) to give genus and species identification.

Extraction and sequencing of ribosomal DNA

DNA was extracted from disrupted cells by a modification of the method described by Kowalchuk *et al* (1997). Bacterial isolates were grown overnight in MRS broth. Samples (1-2 mL) of this culture were centrifuged (10-15 min at 15,000g) in a Beckman Microfuge 18 Centrifuge (Beckman Coulter Inc., Fullerton, CA, USA) to obtain a cell pellet. To lyse the cell pellet, 0.3 g zirconia/silica beads (diameter 0.1 mm; Daintree Scientific, Tasmania, Australia), 0.5 mL of extraction buffer (100 mM Tris-HCl [pH 8], 50 mM EDTA [pH 8], 100 mM NaCl, 1% sodium dodecyl sulphate [SDS]) and 0.5 mL phenol:chloroform:isoamyl alcohol (24:24:1 (v:v:v) Sigma-Aldrich, Australia) were mixed in a 2 mL screw capped microtube. The samples were shaken at 5000 rpm for 30 s in a mini-bead beater (Biospec Products, OK, USA). After centrifugation for 10 min (15,000 g at 4°C), 0.4 mL of the upper layer was removed. DNA was precipitated by addition of 0.6 mL of isopropanol and the mix allowed to stand for 24 h at -20°C. After centrifugation for 10 min (15,000 g), the pellet was washed once with 70% ethanol and allowed to air dry. The dried pellet was dissolved in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]) and stored at -20°C until use in PCR.

Extracted DNA was used as template DNA in the polymerase chain reaction (PCR) to amplify sections of the 16S ribosomal RNA region. The extracted DNA was amplified by PCR using universal primers for the 968 (968f 5'-AACGCGAAGAACCTTAC) and 1401 (1401r 5'-CGGTGTGTACAAGACCC) region, *Escherichia coli* numbering (Bae *et al* 2004). These primers were obtained from Sigma Genosys, NSW, Australia. The PCR reaction mixtures contained 10 mmol l^{-1} Tris HCl (pH 8.3), 50 mmol l^{-1} KCl, 0.2 µmol l^{-1} of each primer, 200 µmol l^{-1} of each dNTP (Roche Diagnostics, Indianapolis, IN, USA), 1.5 mmol l^{-1} $MgCl_2$, 1.25

U of Gold *Taq* DNA Polymerase (AmpliTaq™, Roche Molecular Systems, Branchburg, NJ, USA) and 10 ng of purified template DNA in 50 µl final volume. Amplification was performed under the following programme: initial denaturation at 95°C for 7 min, 10 cycles at 94°C for 30 s (denaturation), 50°C for 30 s (annealing) and 72°C for 45 s (extension), followed by 20 cycles of 94°C for 30 s (denaturation), 50°C for 30 s (annealing) and 72°C for 30 s (extension), with the final extension conducted for 10 min at 72°C. Confirmation of amplicons was done by agarose gel electrophoresis after which they were used for sequencing with the ABI PRISM® BigDye™ Terminators v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA). The products were sequenced at the Ramaciotti Centre for Gene Function Analysis, UNSW, Australia. The resulting sequences underwent DNA similarity searches on NCBI Blast program using sequences retrieved from the Genebank Database (Karlin & Altschul, 1990).

3.2.3 Electron Microscopic Examination

Pure cell cultures, grown in MRS broth (bacteria) or ME broth (yeast) were sedimented by centrifugation (5 minutes at 1 000g). The supernatant was removed and cell pellets were fixed in 5% (v/v) glutaraldehyde in PBS (0.1M, pH 7.2) overnight at room temperature. Cell pellets were then washed three times for 10 min each with PBS (0.1M, pH 7.2). Coverslips (12 x 12 mm) were coated with 0.1% ethylene imine polymer solution (Fluka, Switzerland). Cell suspensions (20 µL) were added to the coverslips and cells were left to adhere to coverslips for 15 min at room temperature. The coverslips were then washed in buffer three times for 10 min each (0.1M phosphate buffer, pH 7.2) and post fixed in 1% osmium tetroxide (OsO_4) in 0.1M PBS (pH 7.2) for 1 h. Following post-fixation, the samples were washed in the same buffer in triplicate for 5 min each and then dehydrated through a graded series of ethanol of 30%, 50%, 70%, 90% and 100% for 10 min each. The samples were then transferred to a critical point dryer (Emitech K850 (ProSciTech, Australia)). The coverslips were then mounted on aluminium stubs and sputter coated with gold (Emitech K550). Slides were observed using a JEOL JSM 7100 Field Emission Scanning Electron Microscope. This work was done in

conjunction with the Microscopy Unit, Department of Biological Sciences, Macquarie University, NSW, Australia.

3.3 RESULTS

3.3.1 Outline of rum production process

Production at the Bundaberg distillery, generally runs 24 hours a day, 6 days a week. It involves sequential operations that are outlined in Figure 3.1. Details of these operations are given in the following sections. Figure 3.2 gives a pictorial presentation of some facilities in the Bundaberg Distillery. Figure 3.3 shows photographs of molasses and dunder.

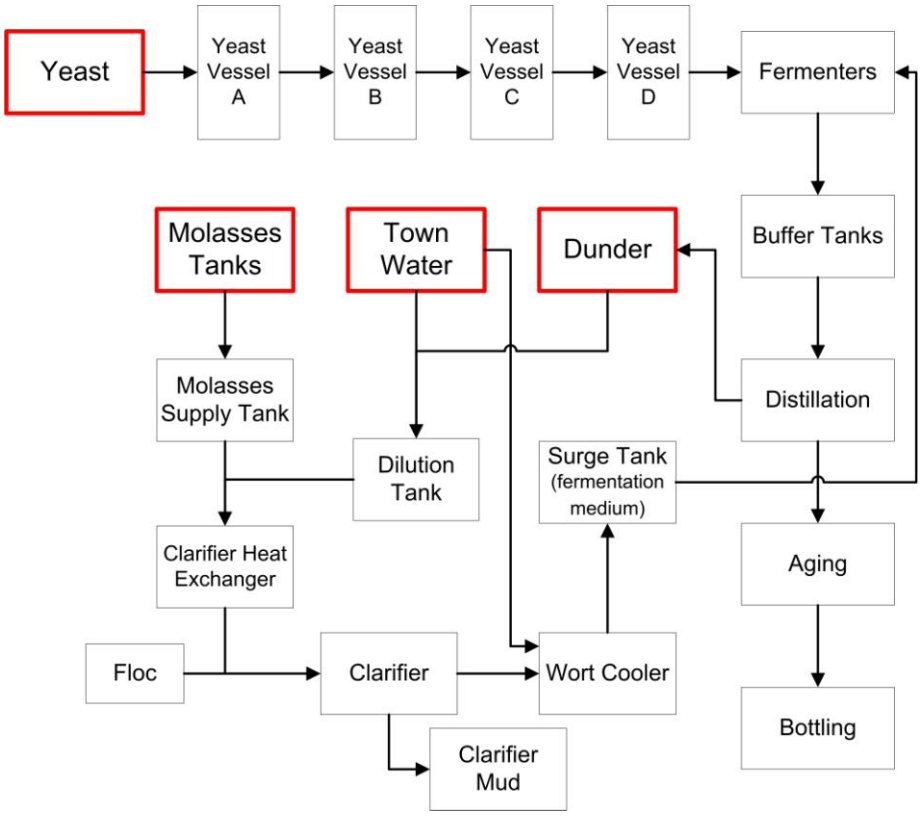


Figure 3.1 Flow diagram of rum production process showing sites of sampling for microbiological analysis as given in Table 3.1

Figure 3.2 Process steps at the Bundaberg Distilling Company. (a) Cane harvest, (b) sugar mill, (c) molasses, (d) molasses storage tank, (e) yeast vessel A, (f) yeast vessels, (g) small fermenters, (h) large stainless steel fermenters, (i) buffer tank, (j) pot still, (k) continuous still, (l) maturation vat, (m) bottling.

3.3.1.1 Molasses and Molasses Preparation

Molasses is the main raw material of the rum production process. It is purchased from an adjacent sugar mill, delivered hot (> 60°C) to the distillery via pipelines and stored in large open tanks (approximately 25m x 15m x 10m) within covered warehouses (Figure 3.2 c, d). The tanks are constructed of concrete. The molasses may remain in these tanks for up to 10 months or longer to provide a constant supply for rum production. The molasses is stored, without temperature control, at ambient temperature that ranges from 20-40°C according to season. Laboratory staff analyse the molasses for fermentable sugars (sucrose, fructose and glucose), total sugars, pH and °Brix on a daily basis. Specific gravity is measured twice weekly. Further chemical analysis is performed sporadically by an external laboratory.

As needed, molasses is pumped from the storage tanks to a clarification vessel where it is diluted with water and dunder to give a final °Brix value of 45-50°. Dunder is added to reach a final concentration of 7.5% of the fermentation medium volume. Flocculant (HISET P730SP, Dai-ichi Kogyo Seiyaku) is added at a ratio of 500 mL per 300 L through a venturi mixer to facilitate the sedimentation of mud and unwanted particulate matter. The mixed materials are heated to 70-80°C and allowed to “stand” for approximately 1h during which the solids sediment. The clarified molasses mixture is then pumped through a heat exchanger to cool it to 35°C. The Brix is further reduced from 50° to 30° by the addition of potable water and the mix is transferred into a surge tank. From here, the molasses, water and dunder mixture (now called fermentation medium) is used to supply the yeast propagation vessels and the main rum fermentations vessels.

Usually, the dunder is added directly from the stills at 100°C. Routine microbiological testing by distillery laboratory staff has shown it to be sterile - viable organisms not detected in 1.0 mL of sample.

Ammonium sulphate, magnesium sulphate and ammonium dihydrogen phosphate are added in early propagation steps to provide additionally nutrients for yeast growth.

Figure 3.3 Raw materials used in the production of rum; molasses (left), dunder (right). Sourced from Bundaberg Distilling Company, Bundaberg Australia

3.3.1.2 Preparation of Yeast

A selected strain of the yeast *Saccharomyces cerevisiae* is used to conduct the fermentation. The strain is stored in several commercial culture collections worldwide. Every 6-12 months, a fresh culture is acquired from these collections from which a pure culture is prepared on MEA agar. It is maintained on slopes of this medium for the next 3 months as the operational culture to propagate yeast inocula for the main rum fermentations. Propagation is a sequential process that aims to adapt the yeast to the rum fermentation medium and to progressively amplify the biomass for large scale fermentation. This process is outlined in Figure 3.1. The early stages of the process (up to and including vessel B) use molasses medium that has been sterilized by heating at 121°C for 20 min. However, medium

sterilization was not possible in the later, larger scale stages. In these cases, the molasses medium, as prepared for the rum fermentation stage, was used. Fermentation medium in the early stages contained added ammonium salts to boost assimilable nitrogen content. The commercial product Fermaid (Lallemand Inc., Canada) was added to the medium in the later stages as an additional source of assimilable nitrogen and vitamins. At each stage of the process, yeast cell counts need to reach 10^5-10^6 cells/mL before transfer to the next stage and these are monitored by microscopic analyses using a haemcytometer. Such analyses also gave an indication of culture purity.

Yeast biomass from the slope is used to inoculate 250 mL of molasses fermentation medium (15°Brix). This culture is incubated for 24 h at 30-32°C after which it is transferred to 2 L of fermentation medium (24°Brix) and incubated at 30-32°C for a further 24 h. This culture is then used in a four stage propagation process whereby it is sequentially inoculated into vessels labelled A to D (Figure 3.1). This propagation sequence is summarised in Figure 3.4.

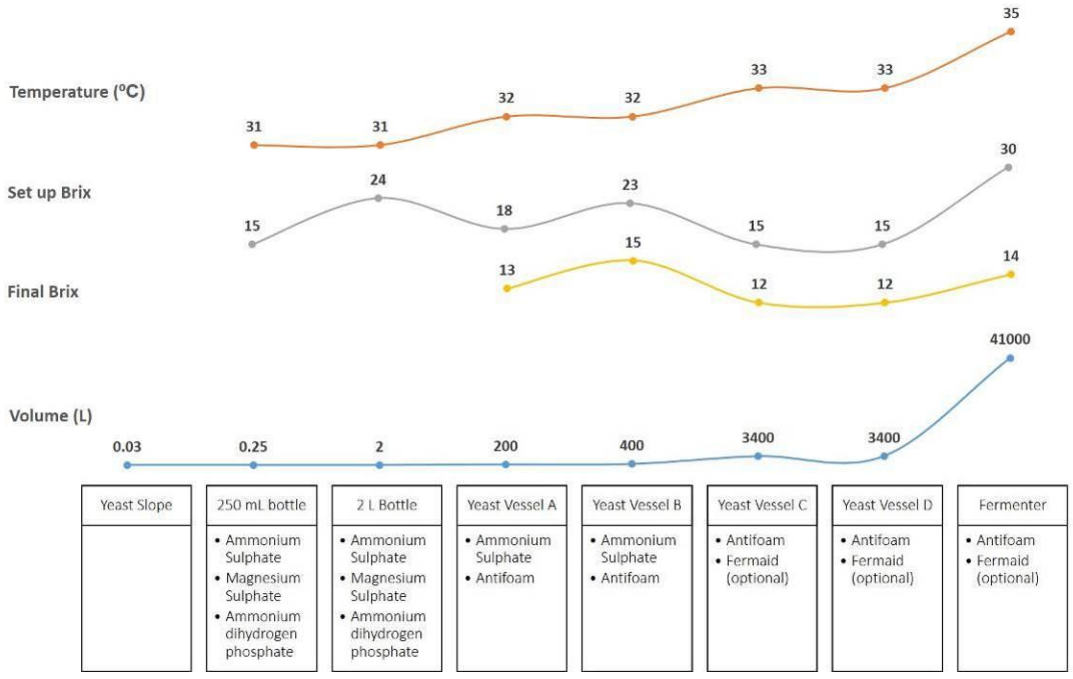


Figure 3.4 Yeast propagation steps undertaken at the Bundaberg distillery

Yeast vessel A consists of 200 L of sterilised fermentation medium (121°C for 20 min at 150 KPa) and is incubated for 12-14 h. The entire volume of this vessel is then transferred, into 400L of sterilised fermentation medium (23°Brix, 32°C) in vessel B. This culture is incubated for 2-3 h, after which it is transferred to vessel C. Incubation in vessel C (3400 L at 15°Brix, unsterilised fermentation medium) is conducted for 1 hour before half of the volume (1700 L) is transferred into vessel D (total volume 3400 L). Conditions in yeast vessel D are similar to those in yeast vessel C. After 1 hour in yeast vessel D, the entire volume is transferred into a fermenter from which samples are used to inoculate the main fermentation tanks. Propagation only lasts for up to 1 hour before being transferred into a fermentation tank.

3.3.1.3 Fermentation

Fermentation medium at 28-32 °Brix is pumped into the fermentation tanks which range in size from small (41000 L) to large (79000 L) tanks (Figure 3.1 and 3.2 g&h). The fermentation medium is mildly agitated during the filling stage (lasts up to 6 h) as the fermenters are filled from the top by pipes that are set against the walls of the tanks and distribute the medium in such a way that it flows in a vortex like manner. This filling operation provides sufficient aeration of the medium to allow a rapid start of the fermentation. Yeast from propagation vessel D is inoculated into the fermentation tanks to give an initial population of approximately 10^5-10^6 cells/mL and an initial °Brix of 28–32. Fermentation is conducted at 30-35°C for 36 h without additional aeration.

3.3.1.4 Buffer Tanks

When fermentation is complete, fermented medium (now called “wash”) from the various fermentation vessels is pumped to one of two 90 000L tanks called buffer tanks (A or B) (Figures 3.1 and 3.2i). These tanks serve to mix the wash from the different fermenters and to provide a continuous supply of fermented wash to the still house. In practice, wash is continuously removed from the buffer tanks but this is not necessarily synchronised with inflow of new wash from any particular

fermenter. Removal of wash from the buffer tanks to the distillation units can stop and start depending on the distillation needs. As a consequence, there is a mixing of new wash with wash already in the buffer tank and, therefore, it is very difficult to know the proportion of newly fermented wash to any fermented wash that was already present in the tank for some period of time. Adding to this imprecision was the irregularity of the cleaning and sanitation of the buffer tanks which was sporadic and, generally, was only done when distillery technicians gauged it necessary due to changes in distillation efficiency. A cessation of distilling operations could cause fermented wash to remain in the buffer tanks for prolonged, indefinite, periods of time.

3.3.1.6 Distillation, Maturation and Packaging

Distillation separates, concentrates and selects for desired volatile compounds produced during fermentation. A two stage distillation process was used at the Bundaberg Distillery. This consists of an initial continuous distillation process, followed by a pot distillation process. Fermented wash from the buffer tanks is pumped to the top of the column or “continuous” still (Figure 3.3k) and flows down through several levels of horizontal trays. Steam is used to heat the liquid in the bottom of the column. Alcohol vapour emerges from the top of the column in a steady stream. The vapour is condensed and the distillate is stored in a “receiver” until there is sufficient volume to be transferred to a pot still (Figure 3.2j). The collected liquid is referred to as “low wines”. Once the pot still is filled, steam is used to heat the liquid in the bottom of the still. The pot still operates similarly to a kettle, with vapour leaving the pot still and condensing to yield a distillate that is transferred to a vessel called the raw rum receiver. The raw rum distillate is then stored in oak vats (Figure 3.2l) for a period of at least 2 years for maturation. After maturation, the rum is packaged for sale (Figure 3.2m).

3.3.1.7 Cleaning and Sanitation

Due to the potentially hazardous environment of volatile vapours, physical cleaning, such as scrubbing and other abrasively dependent cleaning methods were only used in some locations and usually only once the plant had been shut down. These shut downs happened sporadically, usually when efficiency had decreased significantly, and could last anywhere between a couple of hours and days. As a result, most cleaning and sanitation on site was clean-in-place (CIP). CIP cleans consist of computer controlled, highly pressurised nitric acid, caustic, sanitiser (when required) and water rinses. The acid and caustic cleans both occur, at approximately 74 - 77°C, with rinses in between, and the total time for the CIP clean is approximately 2h 45min.

There are cleaning schedules for the Bundaberg distillery. Depending on which piece of equipment or area is needed to be cleaned there are daily, weekly, monthly or ‘as required’ cleans. For example, pipe work relating to the emptying of yeast vessel D to fermenters may be cleaned frequently, (daily) depending on frequency of filling. Yeast vessels are cleaned at the completion of each propagation step, in preparation for the next set of propagation steps, usually daily. Each yeast vessel has an isolated CIP circuit so they can be cleaned individually of each other. Cleaning includes a combination of acid, caustic and sanitiser. Fermenters and associated pipes are cleaned between fermentations, approximately every 36-40 hours. Buffer tanks are rinsed with water weekly however are only thoroughly cleaned sporadically, when a build-up of fermentation sediment occurs and the buffer tank capacity is reduced. The heat exchanger used to heat the fermentation medium post clarification was cleaned only 2-3 times a year and usually only when efficiency is severely reduced (as determined by the production engineers, on a case by case basis, established on expected production efficiency levels).

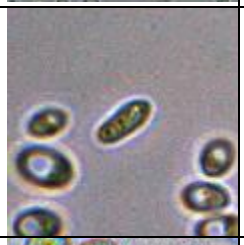
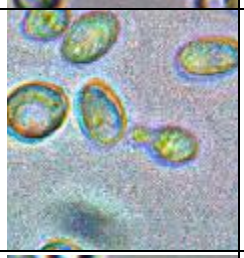
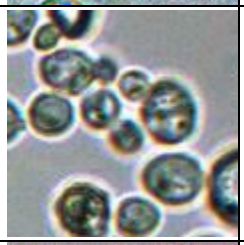
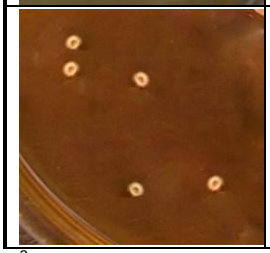
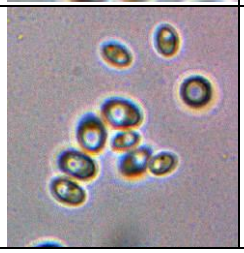
3.3.2 Ecological Investigation of Yeasts and Bacteria throughout the Rum Production Process.

Samples of materials were systematically collected throughout the process chain and examined for their populations of yeasts and bacteria. Predominant yeasts and bacteria were isolated and identified to genus and species. The first part of this section describes the occurrence and growth of yeasts throughout the process, and the second part shows the occurrence and growth of bacteria along the process chain. Results presented in these sections are the means of data obtained from the analyses of duplicate biological samples that were each analysed for microbial populations by plating in duplicate on the appropriate culture medium.

3.2.2.1 Identification of yeasts

Throughout the course of this investigation, five yeast species were isolated on either the MEA or WL agar as evident from their different colony morphologies. These species were *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii, Schizosaccharomyces pombe, Zygosaccharomyces bailii* and *Ogataea thermomethanolica*. Their colony and cellular morphologies as well as identification by sequencing of the rDNA are given in Table 3.2.

Table 3.2. Morphology and sequence identification of yeasts associated with rum production

Colony morphology on MEA	Light microscopy images	Species identification by rDNA sequencing	Accession number	Confirmed identity[a]
		*Saccharomyces cerevisiae*	DQ466538.1	100
		*Schizosaccharomyces pombe*	DQ466539.1	99
		*Ogataea thermomethanolica*	AB200287	89
		*Zygosaccharomyces rouxii*	AJ783431.1	100
		*Zygosaccharomyces bailii*	GI4038807	100

[a] confirmed identity by sequencing of the 26S rDNA (% match according to the BLAST database)

Overall, *S. cerevisiae* was the main yeast isolated and corresponded to the starter culture used by the company to conduct the rum fermentations. Figure 3.5 shows a scanning electron micrograph of this yeast.

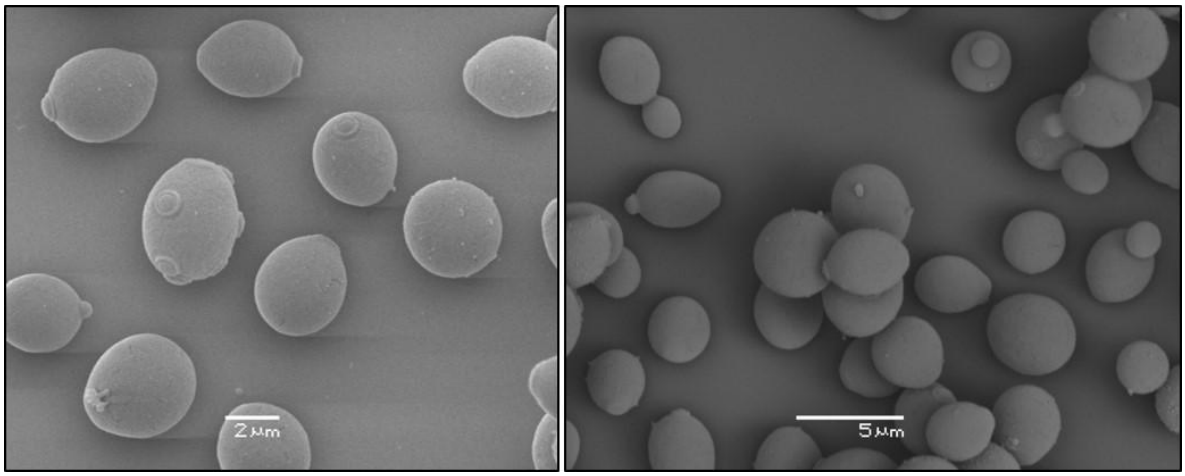


Figure 3.5 Scanning electron microscope image of *Saccharomyces cerevisiae* isolated consistently from the rum distillery (Electron Microscope images obtained at Macquarie University Microscopy Unit, NSW, Australia).

Yeasts in molasses.

Over a four year period, random samples of molasses were taken and examined for the presence of yeasts by culture on plates of MEA and WL agar (Table 3.3). These samples were taken directly from the storage wells or from holding tanks just prior to clarification. Yeast counts were very low at less than 1000 CFU/mL and, on some occasions, no viable yeasts could be detected (< 5 CFU/mL).

Saccharomyces cerevisiae was the most frequently isolated species, being found in 11 out of the 16 samples followed by *Schizosaccharomyces pombe* in six out of 16 samples. *Zygosaccharomyces rouxii* was found in four of the samples while *Zygosaccharomyces balii* (sample 9 at 3.1 x 10^1 CFU/mL) and *Ogataea thermomethanolica* (sample 11 at 1.4 x 10^1 CFU/mL) were found only once each (data not shown in table).

Table 3.3 Occurrence of yeast species in samples of molasses taken at the Bundaberg distillery

Sample number	Sample date	Population (CFU/mL)		
		S. cerevisiae	*Schiz. pombe*	*Zygo. rouxii*
1	10/08/06	nd	nd	nd
2	10/08/06	nd	nd	nd
3	22/01/07	1.1×10^2	0.1×10^1	1.0×10^1
4	09/03/07	0.8×10^1	nd	2.2×10^1
5	31/01/08	nd	nd	nd
6	04/02/08	0.7×10^1	nd	nd
7	10/03/08	1.1×10^2	3.8×10^1	nd
8	10/03/08	2.4×10^2	nd	nd
9	12/03/08	3.8×10^1	2.5×10^1	3.1×10^1
10	12/03/08	2.0×10^2	nd	nd
11	12/03/08	1.4×10^2	3.5×10^1	1.4×10^1
12	12/03/08	2.0×10^2	1.0×10^1	nd
13	09/11/08	0.9×10^1	nd	nd
14	09/11/08	1.3×10^1	0.7×10^1	nd
15	27/04/10	nd	nd	nd
16	27/04/10	nd	nd	nd

nd – not detected, less than 5 CFU/mL

Occurrence and growth of yeasts throughout the rum production process

Preliminary studies in 2006 revealed no detectable yeasts (< 5 CFU/mL) in samples of the raw materials (molasses, town water, dunder) and consequently they were not detected in freshly prepared fermentation medium as taken from the surge tanks just prior to transfer to the fermenters. High populations (10^7-10^8 CFU/mL) were found in the yeast propagation vessels and these consisted of only *S. cerevisiae*, the starter culture. These same high populations of *S. cerevisiae* were found in samples taken from two different fermentation tanks and samples of buffer tanks A and B. No yeasts other than *S. cerevisiae* were detected in these locations. It was concluded from these preliminary trials that rum fermentations at the distillery were conducted by the selected starter culture of *S. cerevisiae* that was routinely propagated at the distillery and inoculated into the fermentation medium.

A more detailed investigation of yeasts throughout the production process was conducted in 2008 and the results are presented in Table 3.4.

Table 3.4 Populations of yeast species throughout the rum production process; conducted in 2008.

Stage/sampling point	Total population		Number of different species
Molasses storage tank	2.0×10^3		2
Molasses supply tank	5.5×10^2		2
Dunder	nd (<10)		-
Dunder and mud line	nd (<10)		-
Dilution tank	nd (<10)		-
Clarifier	nd (<10)		-
Surge tank (50° Brix)	nd (<10)		-
Surge tank (30° Brix)	4.5×10^2		2
Floc	nd (<10)		-
Acid water	nd (<10)		-
Yeast; laboratory propagation	1.6×10^8		1[a]
Yeast vessel A (early 1000h)	5.9×10^5		1[a]
Yeast vessel A (2300h)	2.4×10^8		1[a]
Yeast vessel B before transfer to C	5.1×10^8		1[a]
Yeast vessel C before transfer to D	9.1×10^7		1[a]
	F5[*]	*F9*[*]	
D prior to transfer to fermenter	4.6×10^7	7.1×10^7	1[a]
Fermenter sample after transfer of yeast	1.9×10^7	1.7×10^7	1[a]
6h	6.3×10^7	2.5×10^7	1[a]
12h	7.1×10^7	7.5×10^7	1[a]
18h	6.8×10^7	7.1×10^7	1[a]
24h	4.0×10^7	5.6×10^7	1[a]
36h	6.1×10^7	7.8×10^7	1[a]
Buffer tank A	6.2×10^7		1[a]
Buffer tank B	5.1×10^7		1[a]

1[a] - Yeast species identified as *S.cerevisiae* introduced as starter culture.
F5 & F9[*] refer to Fermenter 5 and Fermenter 9. These fermenters were the two observed for this study. F5 is an example of an old fermenter (smaller in volume 41000L) and F9 is a newer fermenter (larger in volume 79000L).

Two species of yeast were consistently isolated at low populations (approximately 10^3 CFU/mL) from the molasses storage tanks. These were identified as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe.* These species carried over into the molasses supply tank, but were not isolated from the molasses preparation after clarification and transfer to the first surge tank.

The culture of yeast propagated in the laboratory gave a yeast population exceeding 10^8 CFU/mL, from which only *Saccharomyces cerevisiae* was isolated. This population was diluted to about 10^5-10^6 CFU/mL on addition to yeast propagation vessel A, but multiplied to populations greater than 10^8 CFU/mL during subsequent incubation in this vessel (Table 3.4). Such populations and species homogeneity were maintained on subsequent transfer to propagation vessels B, C and D. This population was diluted to about 10^7 CFU/mL on inoculation into the fermenters. Several fermentation tanks are usually inoculated at this stage, and two of these, tank F5 and tank F9 were subsequently monitored for yeast growth (Table 3.4). These tanks represented an older and newer style of fermentation tank. The yeast quickly grew to about 7.0 x10^7 CFU/mL, and remained at such populations until the end of fermentation at 36 h. Similar population data were obtained for both fermentation tanks and for analysis on either MEA or WL agar (Figure 3.6).

Saccharomyces cerevisiae was the only species isolated throughout fermentation. On completion of fermentation, the fermented wash from the different fermenters was pumped into either of two buffer (or mixing) tanks where the yeast population remained at about 5.0 x10^7 CFU/mL and consisted of *Saccharomyces cerevisiae* (Table 3.4).

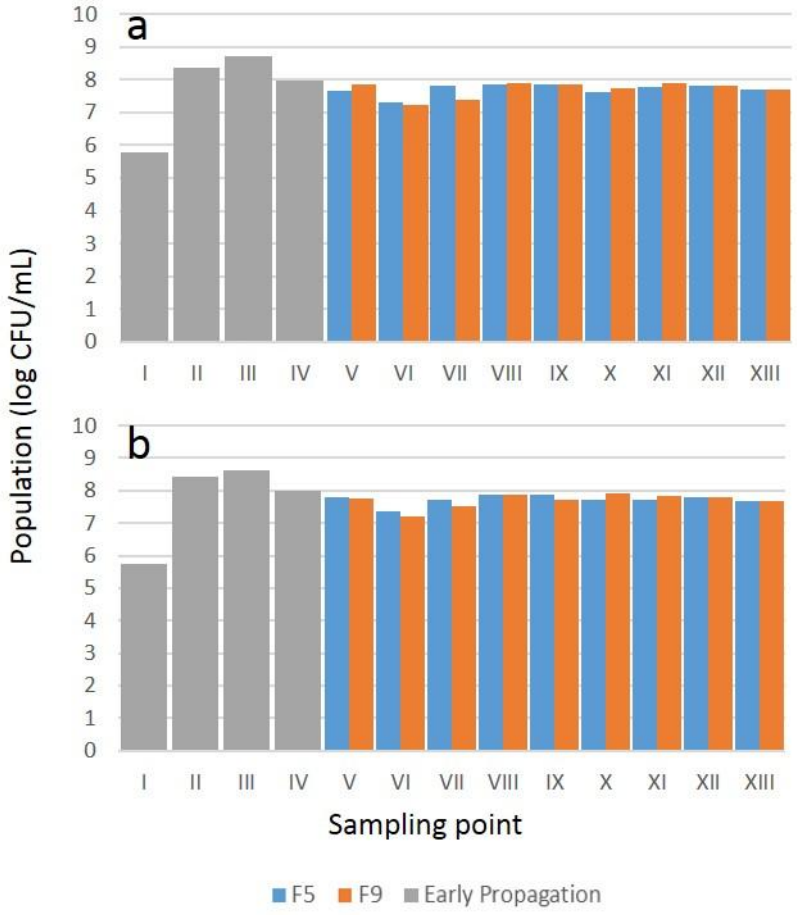


Figure 3.6 Yeast populations during production of rum as determined by culture on, MEA (a), WLA (b). (I) Yeast propagation vessel A early, (II) yeast propagation vessel A before transfer to vessel B, (III) yeast propagation vessel B, (IV) yeast propagation vessel C, (V) yeast propagation vessel D, (VI) 0 h (post transfer from yeast vessel into fermenter), (VII) 6 h, (VIII) 12 h, (IX) 18 h, (X) 24 h, (XI) 36 h, (XII) buffer tank A, (XIII) buffer tank B

After the in depth survey performed in 2008, there was a requirement for a small, targeted ecological survey to understand some critical points from the 2008 survey. The data for yeast analyses are shown in Table 3.5 and confirm the single presence of *S. cerevisiae* in the yeast propagation vessels and buffer tanks at levels of 10^8-10^9 CFU/mL.

Table 3.5 Supporting yeast population data from mini-ecological survey performed in 2010.

Stage/sampling Point	Total population (CFU/mL)	Number of different species
Molasses (2 tanks)	< 5	N/A
Dunder	< 5	N/A
Surge tank – in	<5	N/A
Surge tank – out	<5	N/A
Wort cooler	<5	N/A
Yeast vessel A (early)	2.0×10^6	1
Yeast vessel A (late)	3.5×10^8	1
Fermenter 5 (12 h)	6.3×10^6	1
Fermenter 9 (12 h)	2.5×10^6	1
Buffer tank A	7.7×10^8	1
Buffer tank B	7.5×10^8	1

3.3.2.2 Identification of Bacteria

Bacteria were routinely isolated by plating samples onto MRS, Raka Ray and WLS agar plates from which isolates were obtained and identified. To determine the morphological characteristics of each species, isolates were streaked onto both MRS and WLS agar. This helped to determine how the differing media impacted on the appearance of the colonies.

Morphological and physiological properties and rDNA sequencing were used to characterise and identify the bacterial species. Biochemical testing using API CHL50 test strips (Biomerieux, Durham NC) was conducted to further support identifications. Throughout the course of the ecological surveys, eight different species of bacteria were isolated from various sampling points during the production process. Table 3.6 gives a list of these bacteria as identified with API strips and rDNA sequencing.

Table 3.6 Identification of bacterial isolates from Bundaberg distillery

Species	Identification by rDNA sequencing[a]	Accession number	API CH50 Identity
Lactobacillus plantarum	98.0	GU064445.1	*L. plantarum* (good - 97.7%)
Lactobacillus fermentum	99.0	HM004216.1	*L. fermentum* (good – 96.9%)
Lactobacillus paracasei	99.0	AB330933.1	*L. paracasei* spp *paracasei* (good - 95.0%)
Uncultured *Lactobacillus*	98.0	GQ082129.1	*L. acidophilus* (good - 92.7%)
Lactobacillus brevis	98.0	EF076751.1	*L. brevis* (good - 97.5%)
Lactobacillus oligofermentans	98.0	AY733085.1	Acceptable ID to genus *Lactobacillus*
Bacillus cereus	99.0	DQ 884352.1	Not performed[b]
Bacillus subtilis	97.0	EU016526.1	Not performed[b]
Lactobacillus farraginis	94.0	AB262733.1	*L. buchneri* (very good - 99.9%)

[a] confirmed identity by sequencing of the 16S rDNA (% match according to the BLAST database)
[b] API CH50 specific for lactic acid bacteria

Two bacterial species were repeatedly isolated over the course of the investigation. These were *Lactobacillus fermentum* and *Lactobacillus plantarum*. Figures 3.7 and 3.8 show the colony morphology of these isolates on MRS agar, and cellular morphology as determined by examination with phase contrast microscopy and scanning electron microscopy.

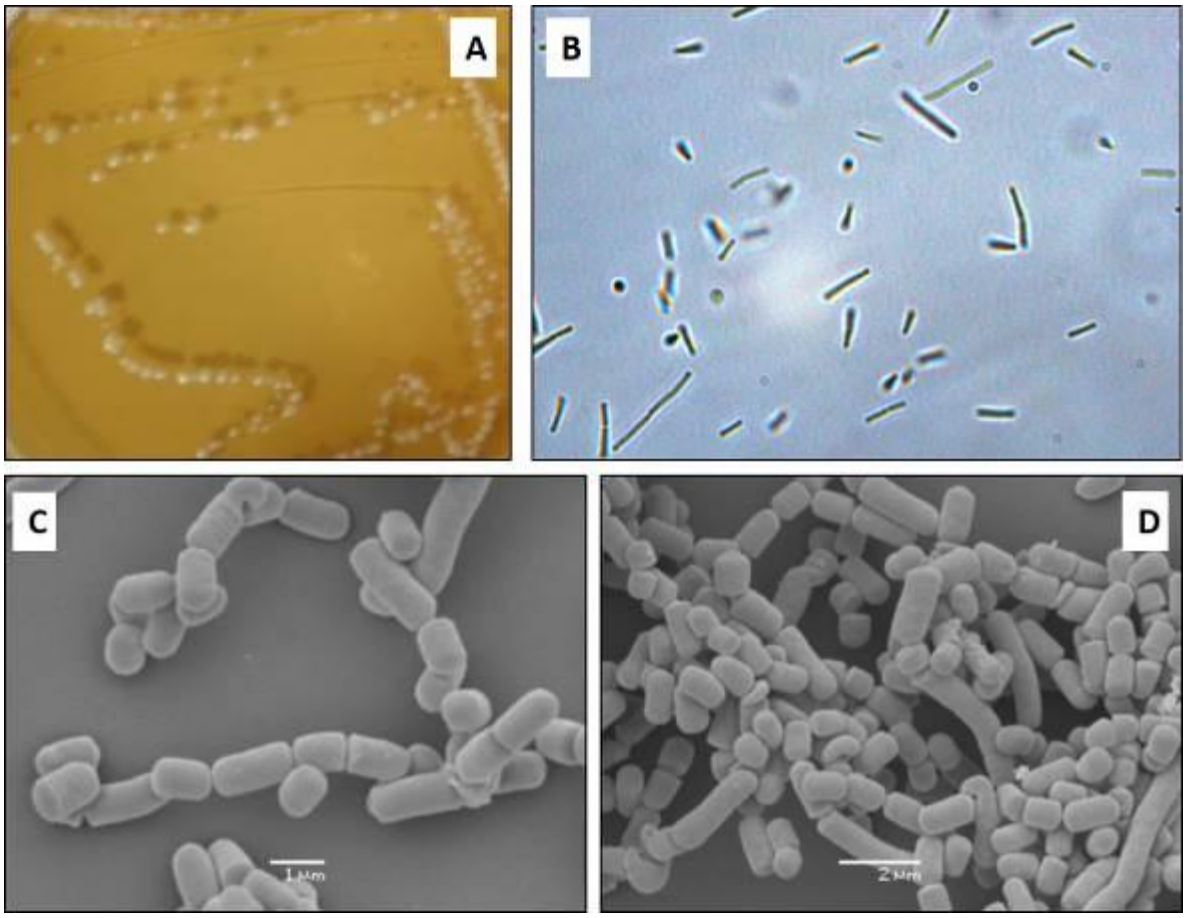


Fig 3.7 *Lactobacillus plantarum* isolated from in-process samples of rum distillery fermentations. (A) appearance on MRS agar, (B) examined under light microscopy (x1000, Leica), (C) and (D) scanning electron microscope. (Electron Microscope images obtained at Macquarie University Microscopy Unit, NSW, Australia.)

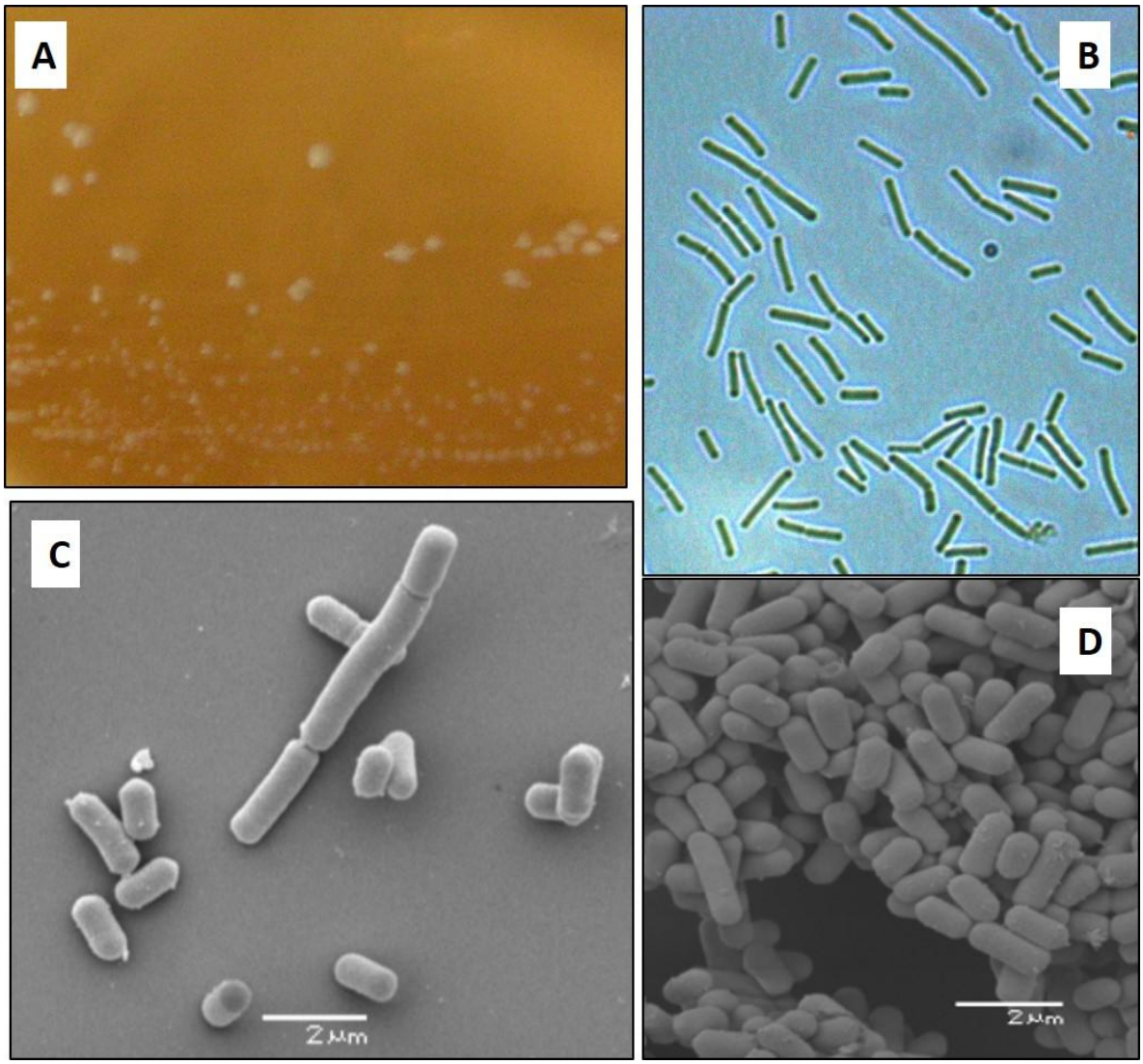


Figure 3.8 *Lactobacillus fermentum* isolated from a rum fermentations. (A) appearance on MRS agar, (B) examined under light microscopy (x1000, Leica), (C) and (D) scanning electron microscope. (Electron Microscope images obtained at Macquarie University Microscopy Unit, NSW, Australia.)

Zymomonas and *Propionibacterium*

Analyses for *Zymomonas* and *Propionibacterium* species were conducted by culturing samples, taken throughout the process during the 2008 and 2010 surveys (Table 3.1), on Universal Beer agar and Sodium Lactate agar, respectively. No isolates of these bacteria were detected (< 50 CFU/mL) in any of the samples. Reference cultures of these species (*Zymomonas mobilis,* ATCC 10988 and *Propionibacterium freundenreichii,* UNSW 035900, respectively) grew on these media as controls to demonstrate that the conditions of cultivation were effective.

Clostridium

Analyses for Clostridium species, were conducted for the 2008 and 2010 surveys, (Table 3.1). Samples were tested using Differential Reinforced Clostridial Agar (RCA) (Difco). No isolates of this species were detected (<1 CFU/mL) in any of the samples. Reference cultures of *Clostridium beijerinckii* (UNSW 060600) grew on RCA as controls.

Bacteria associated with molasses

During 2006-2010, random samples of molasses were taken and examined for the presence of bacteria by culture on plates of MRS, Raka Ray and WLS agar (Table 3.7). These samples were taken directly from the storage wells or from holding tanks just prior to clarification. Bacterial counts were very low, at less than 1000 CFU/mL and, on some occasions, no viable bacteria could be detected (< 5 CFU/mL).

Bacillus subtilis was the most frequently isolated species, being found in 11 out of the 16 samples, followed by *Lactobacillus* spp. in six out of 16 samples. *Bacillus cereus* was found in one of the samples (1.0×10^1CFU/mL). The *Lactobacillus* spp. were not identifiable by API testing and were given as equivalent to an unculturable *Lactobacillus* spp. by rDNA sequencing.

Table 3.7 Occurrence of bacterial species in samples of molasses taken at the Bundaberg distillery

Sample number	Sample date	Population (CFU/mL)		
		B. cereus	*B. subtilis*	*Lactobacillus* spp.
1	10/08/06	nd <5	nd <5	nd <5
2	10/08/06	nd <5	nd <5	nd <5
3	22/01/07	nd <5	3.5×10^2	nd <5
4	09/03/07	nd <5	2.5×10^2	1.4×10^2
5	31/01/08	nd <5	2.5×10^2	1.1×10^2
6	04/02/08	nd <5	2.5×10^2	nd <5
7	10/03/08	nd <5	nd <5	nd <5
8	10/03/08	nd <5	1.5×10^3	1.0×10^1
9	12/03/08	nd <5	2.5×10^2	2.2×10^2
10	12/03/08	nd <5	8.0×10^1	4.0×10^1
11	12/03/08	nd <5	9.0×10^1	nd <5
12	12/03/08	nd <5	nd <5	nd <5
13	09/11/08	nd <5	nd <5	3.0×10^1
14	09/11/08	1.0×10^1	2.0×10^1	nd <5
15	27/04/10	nd <5	4.5×10^2	nd <5
16	27/04/10	nd <5	3.0×10^2	nd <5

nd – not detected, less than 5 CFU/mL

Occurrence and growth of bacteria throughout the rum production process

Preliminary studies in 2006 revealed a complex, and largely unknown, population of bacteria present in the fermentations at the Bundaberg distillery. These studies included examination of samples by phase contrast microscopy, and exploratory plating onto culture media routinely used by the quality assurance laboratory at the Bundaberg distillery.

When examined by phase contrast microscopy, samples of fermenting molasses medium from the fermenters and buffer tanks consistently showed the presence of bacterial cells in addition to yeast cells. Typically, the bacterial populations were approximately 5-10 times less than the yeast population and consisted mainly of long, thin rod-shaped cells. Samples for culture were taken at the following sites; town water supply, dunder, molasses, molasses medium in surge tank, yeast propagation vessel C, fermenter 3, fermenter 8 and buffer tanks A and B. These

samples were examined by spread plate culture on each of the following media - PCA, Raka Ray, MRS and WLS. Using these media, no bacteria (CFU/mL <100) were detected in samples of town water, dunder, molasses, and molasses medium in the surge tank. However, bacteria were detected in samples taken from the yeast propagation vessel C, at populations of 10^4-10^5 CFU/mL and in samples taken from the two fermentation vessels and the two buffer tanks at populations of 10^5-10^6 CFU/mL. It was concluded, from these preliminary studies, that rum fermentations conducted at the distillery consistently exhibited the presence and growth of bacteria in addition to the yeast starter culture (*S. cerevisiae*).

A more detailed investigation of bacteria throughout the production process was conducted in 2008 and the results are presented in Table 3.8. The predominant bacterial species changed as the sample points progressed through the production process. Low populations of *Bacillus* species and the unidentified *Lactobacillus* spp. were found in molasses samples and these carried through into the molasses medium present in the surge tanks. No bacteria were isolated from samples of town water, flocculating agent or dunder used in preparation of the molasses medium. Also, no bacteria were detected in the steps used to prepare the yeast starter culture at the laboratory stage. However, lactic acid bacteria were consistently isolated from the larger yeast propagation vessels in the factory. These included four species, *L. plantarum*, *L. fermentum*, *L. brevis* and the unidentified *Lactobacillus*. Bacteria were not detected in yeast propagation vessel A, initially, but after 12-14 h bacterial populations reached 1.5×10^1 CFU/mL. These populations increased in yeast propagation vessel B to about 10^6 CFU/mL and continued at this level in yeast vessel C. A decline in these populations occurred in conjunction with dilution into yeast vessel D and subsequent transfer to a larger vessel for fermentation. Four species were consistently isolated from these yeast propagation vessels. These were predominantly *L. plantarum* and *L. fermentum*, the species identified as an unculturable *Lactobacillus*, and to a lesser extent, *L brevis*.

Table 3.8 Populations of bacterial species throughout the rum production process; conducted in 2008

Sample point	*Species isolated*	*Average population CFU/mL*	
Molasses	*Bacillus subtilis*	1.5×10^3	
	Lactobacillus spp.	9.0×10^2	
	Bacillus cereus	1.0×10^1	
Dunder	none	nd (<10)	
Dunder and mud line	none	nd (<10)	
Dilution tank	none	nd (<10)	
Clarifier	*Bacillus subtilis*	1.6×10^1	
	Lactobacillus spp.	nd (<10)	
	Bacillus cereus	2.2×10^1	
Surge tank (50° Brix)	*Bacillus subtilis*	3.0×10^1	
	Lactobacillus spp.	nd (<10)	
Surge tank (30° Brix)	*Bacillus subtilis*	4.5×10^1	
	Lactobacillus spp.	nd (<10)	
Floc	none	nd (<10)	
Acid water	none	nd (<10)	
Yeast starter culture	none	nd (<10)	
Yeast propagation	none	nd (<10)	
Yeast propagation vessel A/B		**A**	**B**
	Lactobacillus plantarum	1.3×10^1	8.1×10^4
	Lactobacillus fermentum	nd (<10)	1.1×10^4
	Lactobacillus brevis	nd (<10)	1.3×10^4
	Lactobacillus spp.	nd (<10)	1.3×10^3
Yeast propagation vessel C/D		**C**	**D**
	Lactobacillus plantarum	1.9×10^6	1.0×10^5
	Lactobacillus fermentum	5.1×10^5	3.2×10^4
	Lactobacillus brevis	nd (<10)	nd (<10)
	Lactobacillus spp.	1.5×10^5	3.0×10^3
Fermentation vessels – Early (0-12 h)		**F5**	**F9**
	Lactobacillus plantarum	2.2×10^6	1.1×10^7
	Lactobacillus fermentum	1.4×10^6	2.6×10^6
	Lactobacillus brevis	1.6×10^4	5.0×10^4
	Lactobacillus spp.	3.0×10^4	1.2×10^5
Fermentation vessels – Late (18 – 36 h)	*Lactobacillus plantarum*	3.7×10^6	3.6×10^7
	Lactobacillus fermentum	2.5×10^5	4.0×10^6
	Lactobacillus brevis	nd (<10)	3.5×10^5
	Lactobacillus spp.	5.0×10^4	5.0×10^5
Buffer tanks		**A**	**B**
	Lactobacillus plantarum	1.6×10^7	2.5×10^6
	Lactobacillus fermentum	5.0×10^6	4.5×10^6

Lactobacillus plantarum and *L. fermentum* were consistently isolated from the molasses medium throughout the fermentation process, reaching populations of 10^6-10^7 CFU/mL or slightly higher in the case of *L. plantarum.* Such populations carried over into the buffer tanks (Table 3.8). Their contributions are shown in more

detail for two different fermentation vessels in Figure 3.9 (a, b, c, d). Similar population data trends were obtained for culture on either WLS or MRS agar. The unidentifiable *Lactobacillus* spp. and *L. brevis* were also prevalent throughout the fermentation process, but their populations were generally lower (10^4 - 10^6 CFU/mL) and they were not as consistently isolated, as evident in Figure 3.9 (e, f, g, h). Also, the two culture media, MRS and WLS agar, did not always give similar population data for these two species. Their less frequent presence and lesser populations reflected in their inconsistent detection in samples taken from the buffer tanks (Fig 3.9).

Lactobacillus parcasei, *Lactobacillus oligofermentans, Lactobacillus farraginis* and *Lactobacillus buchneri* were randomly isolated from the fermenting molasses medium but at low populations (< 4.1 x 10^4 CFU/mL).

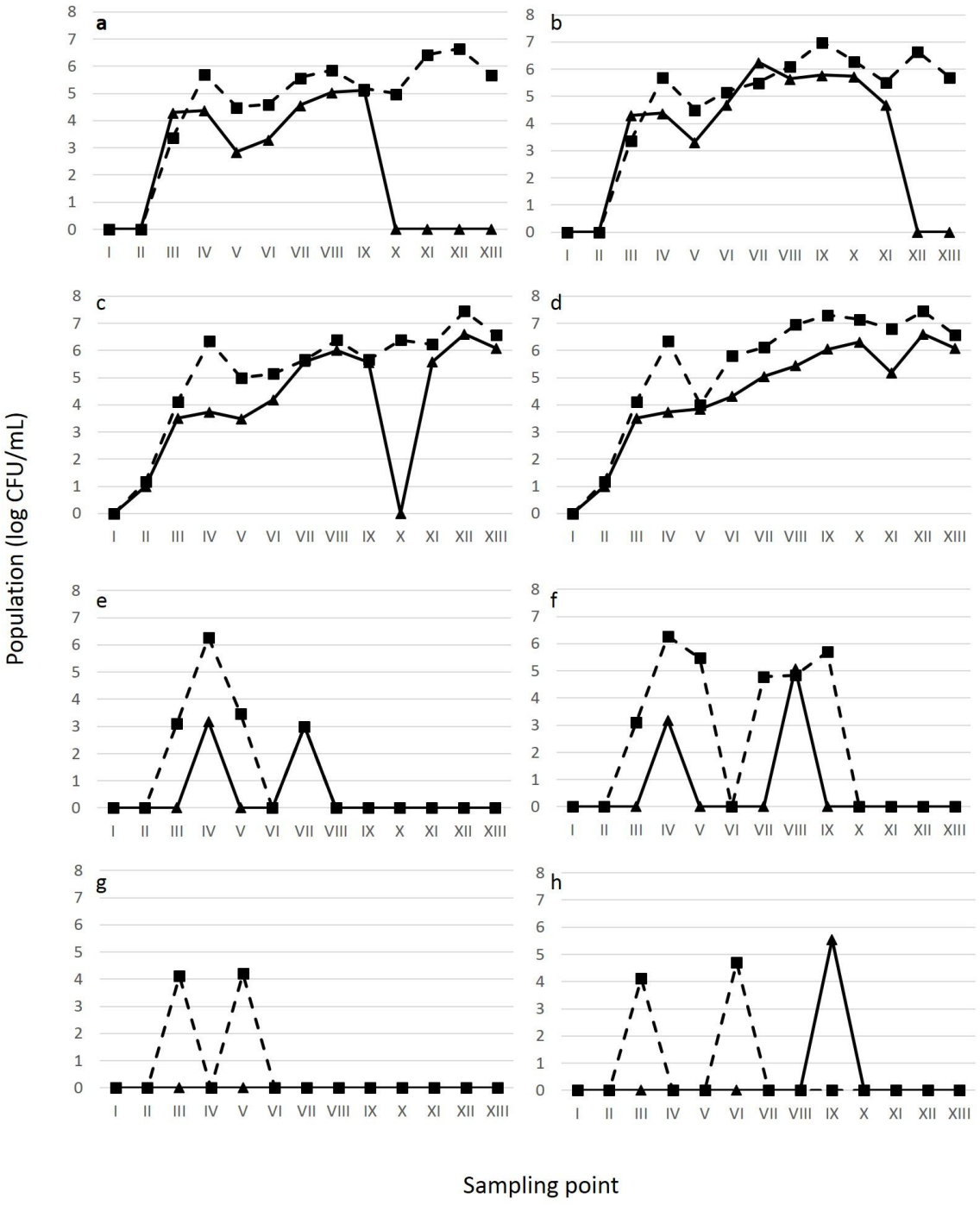


Figure 3.9 Changes in the populations of individual bacterial species (a,b *L. fermentum*, c,d *L. plantarum*, e,f *Lactobacillus* spp. g,h *L. brevis*) during fermentation of molasses, on two different media (■) MRS (▲) WLS from a small fermenter (a,c,e,g) and large fermenter (b, d, f, h). (I) yeast propagation vessel A early, (II) yeast propagation vessel A before transfer to vessel B, (III) yeast propagation vessel B, (IV) yeast propagation vessel C, (V) yeast propagation vessel D, (VI) 0 h (post transfer from yeast vessel into fermenter), fermenter at (VII) 6 h, (VIII) 12 h, (IX) 18 h, (X) 24 h, (XI) 36 h, (XII) buffer tank A, (XIII) buffer tank B

After the in depth survey performed in 2008, there was a need for a targeted survey of a few critical points in the production process. The data for bacterial analyses are shown in Table 3.9. These findings confirmed those obtained in the earlier investigations of 2008 and 2006. These were: absence of bacteria in dunder samples; presence of *Bacillus* and *Lactobacillus* in samples of molasses medium from the surge tank; presence of *L. plantarum* and *L. fermentum* in the yeast propagation system; and prevalence of *L. fermentum*, *L. plantarum* and to a lesser extent the unidentifiable *Lactobacillus* spp. in the fermentation process as evident from data obtained from buffer tanks

Table 3.9 Supporting bacterial population data from mini ecological survey (2010)

Stage/sampling Point	Species isolated	Total population (CFU/mL)
Dunder (after still)	N/A	nil
Dunder (after clarifier)	N/A	nil
Wort cooler	*Bacillus subtilis*	5.0×10^1
	Lactobacillus spp.	
Surge tank (in)	*Bacillus subtilis*	2.4×10^2
	Lactobacillus spp.	6.0×10^1
Surge tank (out)	*Bacillus subtilis*	4.2×10^2
	Lactobacillus spp.	3.0×10^1
Yeast propagation Vessel A (early)	N/A	nil
Yeast propagation Vessel A (late)	*L. fermentum*	1.3×10^4
	L. plantarum	4.1×10^4
Buffer tank A	*L. fermentum*	7.3×10^6
	L. plantarum	3.5×10^6
	Lactobacillus spp.	2.0×10^5
Buffer tank B	*L. fermentum*	1.0×10^7
	L. plantarum	2.2×10^6

3.4 DISCUSSION

This chapter reports the first systematic investigation of the microbiology of rum production in an Australian rum distillery. Its main aim was to understand which microbial species are predominant during the molasses fermentation, how these species grow and develop throughout the process and the likely sources of these microorganisms. With this basic ecological information, quality assurance procedures could then be refined and developed to better manage the process.

The Bundaberg Distilling Company

Production of rum first started at The Bundaberg Distilling Company, Bundaberg, Queensland in 1888. It was set up and run by the operators of local sugar cane processing mills as a means to manage waste molasses from sugar production (Kerr, 1983). As rum production was not a main business focus of the sugar cane farmers and millers, the distilling operation evolved and developed somewhat empirically over the years to become the largest rum producing operation in Australia. In 2000, it was purchased by the British based multinational, alcoholic beverage company, Diageo plc. Presently, the Bundaberg Distilling Company produces heavy body, dark rums that are highly appreciated by local consumers for their unique flavours, but are less valued in an international context (Broom, 2003). Approximately half a million 9L cases valued at $200 million are produced annually (Main, 2010). Bundaberg Rum is an iconic brand name to Australian consumers, recently celebrating 125 years of production (Kerr, 1983).

Future development and expansion of the distillery requires a more thorough understanding of the science and technology of the overall operation so systems can be improved or developed to better manage production efficiency and product quality. The company is aware that its product has unique character in relation to rums, globally, but is uncertain as to what factors lead to this property. It is with this background that the microbiological investigations reported in this chapter were undertaken.

Production process

The basic process followed those described in the literature for rums produced using sugar cane molasses as the main raw material (Anson, 1971; Kampen, 1975; Lehtonen & Suomalainen, 1977; Nicol, 2003; James, 2008). Dunder was added to the molasses fermentation medium and the company used a fermentation process that relied on inoculation with a specific strain of *Saccharomyces cerevisiae*. The overall operation, plant equipment and quality assurance practices had evolved over many years. Consequently, there was a mixture of old and new equipment, (such as the fermentation vessels), myriads of pipelines to accommodate new additions as the production expanded, staff who had been associated with the company for many years, and entrenched quality assurance procedures. Although basic microbiological testing was routinely done, the staff had no formal microbiological training or qualifications. There was an assumption that the fermentation process was due to the inoculated yeast because a starter culture of this yeast was used in conjunction with heat treatment (80°C) of the molasses medium for fermentation and the regular use of CIP systems.

Molasses

The stored molasses at the Bundaberg distillery had an initial °Brix of approximately 84°Brix, water activity (A_w) of 0.7 - 0.8, fermentable sugar content of 60-80% and a pH 5.1 – 5.7. This is consistent with literature values (Lehtonen & Suomalainen, 1977; Kampen, 1975, Nicol, 2003). The molasses was stored in large in-ground pools/tanks with periodic topping up from external suppliers as deemed necessary, due to production requirements. These pools were open to the atmosphere from where external contamination could occur. The tanks were rarely emptied and cleaned, neither were the associated pipes and pumps. Culturable microbial populations were very low ($<10^3$ CFU/mL), with many molasses samples giving no detectable microorganisms on various occasions. Possibly, those samples were obtained immediately after a topping up operation when the temperature of the incoming molasses would be high (>60°C) as a consequence of the sugar processing operation.

Studies performed by Browne (1929) over a 14 year period, failed to isolate any yeast, moulds or bacteria from molasses samples. Other researchers found low populations of bacteria (Fahrasmane & Ganou-Parfait, 1998) similar to those obtained in this study. In attempts to improve the recovery of culturable organisms from the molasses samples (compared with results obtained on PCA, MRS, Raka Ray, WL and WLS), some samples were simultaneously cultured on PCA, into which either 5% w/v sucrose, 5% w/v molasses or 5% v/v dunder had been incorporated. However, trials with these media did not improve the growth of the bacteria on the plates or lead to the isolation of additional species.

The molasses tanks, with storage over many years without cleaning, represent a unique microbial ecosystem, presenting a specific environment of very high sugar concentration, low A_w, very little available nitrogen sources and the presence of microbial inhibitors (Bluhm, 1983; Curtin, 1975; Lehtonen & Suomalainen, 1977; Nicol, 2003). Such conditions are generally hostile to the survival and growth of microorganisms and explain the very low populations observed in this study and by others (Fahrasmane & Ganou-Parfait, 1998).

Yeasts have been frequently isolated from stored molasses, although at low levels $< 10^3$ CFU/g. As might be expected, the most frequent isolates have been osmo-tolerant species of *Zygosaccharomyces* and *Schizosaccharomyces* (Hall *et al*, 1935; Parfait & Sabin, 1975; Bonilla-Salinas, 1995) along with *S. cerevisiae* which is also known to tolerate high concentrations of sugars (El-Tabey Shehata *et al*., 1959; Bonilla-Salinas, 1995). Similar data were found during this study (Table 3.3).

The bacteria of molasses used for rum production have not been systematically studied. The few studies available report very low populations (10^2 - 10^3 CFU/ mL) and random isolations of *Clostridium saccharolyticum*, *Propionibacterium jensenii, Lactobacillus fructivorans, Leuconostoc paramesenteroides* and *Lactobacillus plantarum* (Hall *et al*, 1935; Ganou-Parfait *et al*, 1989; Fahrasmane & Ganou-Parfait, 1998; Todorov & Dicks, 2004). The most frequently isolated bacteria from molasses in the present study were species of *Bacillus* - especially *B. subtilis*. This is not unexpected as it is a spore forming organism that could withstand the heat

processing of the sugar cane, and the spores are likely to survive in stored molasses (Owen, 1911). Previous research into molasses based rum fermentations did not find *Bacillus* species in the molasses raw material. However, *Bacillus* species have been isolated from molasses fermentation media used in the production of rums (Ganou-Parfait *et al*, 1987; Fahrasmane *et al*, 1988; Ganou-Parafit *et al*, 1989).

Although not consistently found, an unidentifiable species of *Lactobacillus* was often isolated from molasses samples (Table 3.7). It was the only species of lactic acid bacteria found in such samples and could be part of an indigenous flora that has developed over time in the molasses storage tanks of the Bundaberg distillery.

Molasses is a complex and unique environment, consequently, there may be species present that cannot be isolated by standard cultural procedures. Such species might be detected by using non-culture based, molecular technologies such as PCR-DGGE or the more recently developed high throughput sequencing technologies that are now being applied to study the microbiology of foods and beverages (Amann *et al*, 1995; Muyzer & Smalla, 1998; Torsvik *et al*, 1998; Rappe & Giovannoni, 2003; Ercolini, 2004; Tyson & Banfield, 2005; Bokulich & Mills, 2012). Facilities for the use of these technologies were not available at the time the research for this project was conducted and further research applying such methods will be needed to better understand the microbial ecology of molasses.

Indigenous microflora present in the molasses could impact on rum quality and flavour, especially if they grow during molasses storage. As mentioned previously, molasses is a hostile environment for microorganisms and naturally restricts microbial growth. The low populations of microorganisms found in molasses in this study are unlikely to have a significant metabolic impact on molasses composition and quality. The molasses storage wells/pits at the Bundaberg distillery were housed within large buildings (sheds) to protect them from the external environment. Nevertheless, the surface of the pits remained exposed to the atmosphere within these housings. Within the company records, there were reports where water had leaked onto the surface of the molasses pits during extreme

weather conditions such as strong storms and tropical rainfall. These circumstances are likely to create micro-environments such as diluted molasses "hot spots" where the A_w would be decreased and the resultant growth of microorganisms could be significant. In such cases, the microbial growth could produce metabolites with the potential to impact on rum flavour and quality. Consequently, monitoring of molasses storage conditions would be an important critical point to be incorporated into the company quality assurance and HACCP management systems.

Preparation of Molasses Fermentation Medium

The aim of molasses preparation was to produce a fermentation medium that had the appropriate concentrations of fermentable sugars and to inactivate contaminating microorganisms present in the molasses. Molasses was diluted with potable water and dunder to a final value of 30°Brix for the fermentation mash. This would consist of approximately 15-20% fermentable sugars that would give a fully fermented product of approximately 7-10% ethanol (Bundaberg in-process documentation, 2009). For the Bundaberg distillery, the diluted molasses incorporated dunder at a final concentration of 7.5%, and the mix was clarified by flocculation and then heated at 70-80°C and left to "stand" for approximately 1 h.

Culturable yeasts and bacteria were not detected in samples of the dunder, sourced directly from the distillation process, and they were not detected in the flocculating agent added to the mixture. After clarification and heating, culturable microorganisms were detected at low populations in the molasses fermentation medium. Only *Bacillus* species at 10^1-10^2 CFU/mL were detected at this stage and this is consistent with their presence in molasses and heat resistance due to the production of endospores. After this stage, the molasses medium was passed through a heat exchanger to cool it down to 50-60°C and then further diluted with potable, UV-sterilized water to give the final Brix value of 30°Brix in the surge tank. Dilution at this stage facilitated the earlier clarification operation, as a more concentrated mixture was used at that point and this avoided the processing and heating of much larger volumes in that operation. However, samples taken from

this surge tank gave low populations of yeast (10^{2}-10^{3} CFU/mL), *Bacillus* species (10^{1}-10^{2} CFU/mL) and the *Lactobacillus* spp. (2010 data) suggesting contamination from unclarified molasses. After the surge tank, the molasses medium was used in the fermentation tanks and in the yeast propagation stages.

Yeast and propagation process

The Bundaberg distillery used a specific strain of *S. cerevisiae* which was stored in several commercial culture collections located around the world. These collections routinely monitored and checked species and strain purity and homogeneity. This ensured the security and continued availability of this critical microorganism. As such, the Bundaberg distillery could be confident that every starter culture provided by the external culture collection was the same strain of *S. cerevisiae* with its specific and desired characteristics – thereby, maintaining consistency in product character and flavour, and minimising the risks of spoilage and failed fermentations (Verstrepen *et al* 2003). The frequency (6-12 months) of renewal of the starter culture from the culture collection, means that the iterations of propagation will not be too far removed from the stock starter culture. This minimizes chances of contamination and evolutionary change. In controlled fermentations, the introduction of a starter culture is undertaken to provide a yeast strain which will impart desirable characteristics to the ferment (Fleet, 2008). This starter culture is added at populations high enough to out-compete any wild yeast present in the raw materials e.g. molasses. This was seen in the Bundaberg distillery where populations of wild yeast were not detected during the propagation and fermentation stages.

The propagation process was a series of stepwise procedures aimed to nurture the starter culture from the storage slope to growth in a chemically and nutritionally complex fermentation medium to high populations of 10^{6}-10^{8} CFU/mL. It involved a laboratory phase where the yeast was cultured in media that had been sterilized by autoclaving (Figure 3.4). For scale up to larger volumes in vessels A-D, molasses medium from the surge tank was used. Fermaid, ammonium sulphate, magnesium sulphate and ammonium dihydrogen phosphate were added, usually during early

propagation steps, to provide extra nitrogen sources and key nutrients and vitamins for yeast growth and survival. Analyses of samples from the various stages of propagation during the 2006, 2008 and 2010 investigations consistently showed the presence of only *S. cerevisiae* at populations of 10^6-10^7 CFU/mL or sometimes higher. Therefore, it may be concluded that, with respect to the yeast, the propagation system used at the distillery was performing correctly. It is assumed that the isolates of yeast obtained throughout the process and identified as *S. cerevisiae* by rDNA sequencing were the same strain. Further research using molecular methods to determine strain homogeneity, such as examining restriction patterns, chromosomal profiles or mtDNA restriction analysis (Fernandez-Espinar *et al*, 2001) would be needed to confirm this assumption.

Unexpectedly, bacteria were found in samples taken throughout the yeast propagation process. They were not detected in samples taken from the propagation stages prepared in the laboratory where autoclaved media were used. However, they first became evident in yeast propagation vessels A and B that contained heat treated molasses fermentation medium, as prepared for the fermentation process. It was generally assumed by distillery staff that this medium would be sterile. However, this study showed this not to be the case, as bacteria were present in samples taken at this stage for both the 2008 and 2010 investigations. These bacteria were not *Bacillus* species as might be expected. Rather, they were a mixture of *Lactobacillus* species, but predominantly *L. plantarum* and *L. fermentum*, which increased in populations to about 10^5-10^6 by the end of the propagation stages. Consequently, the starter culture used to inoculate the fermentation vessels was not a pure culture of the selected strain of *S. cerevisiae* and contained significant populations of up to four species of *Lactobacillus*. Knowing and understanding the source of this bacterial contamination is important and will be discussed in a later section.

Fermentation Process

Before inoculation, the molasses fermentation medium showed non-detectable or low populations (approx. 10^2 CFU/mL) of yeasts. After inoculation from propagation

vessel D, the initial yeast population was approximately 10^7 CFU/mL and only *S. cerevisiae* was found. This species grew during fermentation and was the only species isolated throughout the fermentation and at the end of fermentation. Maximum populations of 10^7-10^8 CFU/mL were achieved during fermentation. These population data are consistent with what has been reported previously for starter culture inoculated, molasses rum fermentations (Arroyo, 1945a, b). Any contaminating yeast species (e.g. *Schizosaccharomyces pombe* or *Zygosaccharomyces* species) that might have originated from the molasses were overwhelmed by the inoculation process and did not establish themselves during fermentation. Also, both *Schizosaccharomyces pombe* and the *Zygosaccharomyces* species have much slower growth rates than *S. cerevisiae* (Lehtonen & Suomalainen, 1977, Fahrasmane *et al*, 1988) and this would contribute to their inability to compete with *S. cerevisiae* during these rum fermentations.

It may be concluded from the yeast analyses, that the inoculated strain of *S. cerevisiae* is the principal yeast responsible for the rum fermentations conducted at the Bundaberg distillery. Its biochemical activities and particular profile of secondary metabolites will have a strong impact on the final quality and flavour of the rum. Although the rum from this distillery may be considered as a heavy style product, there was no quantifiable contribution from *Schizosaccharomyces pombe*, which is frequently associated with the fermentation and production of these particular rum styles (Fahrasmane & Ganou-Parfait, 1998; Lehtonen & Suomalainen, 1977).

Lactic acid bacteria were consistently isolated from the molasses rum fermentations conducted at the Bundaberg distillery. They were found at initial populations of 10^4 – 10^6 CFU/mL at the commencement of fermentation and this is consistent with their presence in the yeast starter culture after propagation as discussed previously. Thereafter, they grew throughout fermentation, reaching maximum populations of 10^7 – 10^8 CFU/mL. The most prevalent species associated with the fermentation were *Lactobacillus plantarum* and *Lactobacillus*

fermentum, but two other species, an unidentifiable *Lactobacillus* species and *Lactobacillus brevis* also contributed to the process, but less consistently and at lower overall populations.

It is evident from the onsite, 2006, 2008 and 2010 ecological surveys of the rum fermentation process that lactic acid bacteria are an integral, but uncontrolled part of the overall operation at the Bundaberg distillery. The extent to which they grow during fermentation (populations of 10^7 – 10^8 CFU/mL) is quantitatively significant and their biochemical activities are most likely to impact on process efficiency and the flavour and quality of the rum product. Because of this importance, further studies on the association of these bacteria with the process will be presented and discussed in Chapters 4 and 6.

As discussed in Chapter 2, sporadic studies have reported the association of bacteria with molasses based rum fermentations (Chapter 2, Table 2.8). Organisms of particular concern in these early studies were species of *Clostridium* and *Propionibacterium,* with only a brief mention of lactic acid bacteria (Hall, *et al* 1935; Fahrasmane *et al.* 1988; Ganou-Parfait *et al*., 1989). Such bacteria were considered to have the potential to positively or negatively impact on product flavour and quality (Allan, 1906; Fahrasmane *et al.* 1988). Despite this significance, systematic studies of the contribution of bacteria to rum fermentations have not been reported. The data presented in this Chapter represent the first detailed investigation of the contribution of bacteria to molasses based rum fermentations and show a definitive presence of lactic acid bacteria. In this context, the findings are consistent with those for cachaça fermentations (Schwan *et al*., 2001; Duarte *et al.*; 2011) and whisky fermentations (Simpson *et al.,* 2001; van Beek & Priest, 2000, 2002 & 2003; Cachat & Priest, 2005) which report similar associations of lactic acid bacteria and conclude the potential of these species to impact on process efficiency and product quality.

Apart from *Bacillus* species, no bacteria other than lactic acid bacteria were found in the Bundaberg distillery molasses or fermentations despite using specific media and culture conditions to monitor the possible presence of *Clostridium*,

Propionibacterium and *Zymomonas* species, as mentioned in some early studies (Fahrasmane *et al.* 1988; Ganou-Parfait *et al*., 1989). In particular, *Clostridium saccharolyticum* has previously been isolated from rum fermentations and has, along with *Bacillus* species (*B. butyricus* and *B. amylobacter*), been linked to the development of important flavour volatiles (Allan, 1906; Hall *et al.* 1935, Ganou-Parfait *et al*, 1989). The contribution of *Clostridium* species to the production of volatile acids (acetic, butyric, caproic, heptanoic and propionic acids), which are precursors of some esters considered essential for rum aroma and flavour, has previously been postulated (Hall *et al.,* 1935). These esters are of particular importance in the dark, heavy style rums. The absence of these bacterial species from the fermentations at the Bundaberg distillery, which produces such rums, is of note.

Cleaning and Sanitation

Although the Bundaberg distillery implemented a well-established, CIP program at the completion of each fermentation cycle, there were some locations and sections of pipe work where cleaning and sanitation were not systematically applied. As mentioned previously, the molasses storage pits and associated pumps and pipe work were rarely cleaned. Provided there was no “infection” within these pits, this would not present a hazard since the molasses is heat treated at the dilution and preparation stage. Ideally, all locations after this stage should be subject to regular and effective cleaning and sanitation. However, this was found not to be the case. As the operation expanded and developed over the years, it was inevitable, that added lines, and pipework, created areas that escaped the CIP system or reduced the CIP effectiveness. After onsite inspections, several locations were identified where the CIP system was not active or regularly applied. These included a secondary dunder storage vessel and associated pipework located near the molasses clarifier tank, various heat exchangers associated with molasses medium preparation, and pipework associated with the yeast propagation vessels and feeding lines to inoculation ports of the fermentation tanks, junctions or “elbows” within the pipe work, provided “dead spaces” with reduced fluid movement. With

the exception of the secondary dunder storage tank, these critical zones were located after the molasses had been diluted and heated, but prior to the fermentation tanks (Figure 3.1). The location of these sites would favour the continued growth of adequate populations of indigenous bacteria that repeatedly “inoculate” the prepared fermentation medium.

There were some locations that were only cleaned “as required”, based on the judgements and expertise of production staff. These decisions were linked to production efficiency and the need to minimize any downtime due to cleaning. Such locations included buffer tanks, clarifier and heat exchangers. For example, the buffer tanks were not cleaned on a daily basis, because they fed the distillation units on a continuous, 24 hour basis. Heat exchangers were often only cleaned at the time they became ineffective due to blockages caused by sediment of mud and scale. These blockages also caused leakages and cross contamination (visible when cooling water on one side of the exchanger became discoloured from hot molasses or dunder on the other side). As a consequence, heat exchangers were monitored by recording pH and colouration of cooling water. Cooling water was continuously circulated via heat exchangers through-out the production facility, as a means of conserving energy and minimising production costs. Thus, heat recovered from the distillation units and dunder storage vessels was used to heat the molasses for preparation of the molasses fermentation medium and then the water was chilled to cool the molasses fermentation medium to the temperature required for fermentation. The same water was continuously recycled throughout the entire production system using a network of heat exchangers.

Quality Assurance Program

The Bundaberg distillery had an onsite quality assurance laboratory. This laboratory was responsible for yeast propagation, raw material testing, checking production efficiency and sensory testing. The staff had no formal qualifications in microbiology and their skills were mostly based in analytical chemistry and company experience passed down over the years. Process efficiency was mostly monitored by regular chemical testing such as the concentration of sugars,

(sucrose, fructose and glucose) in the molasses and sugar and ethanol content in the fermented molasses and dunder. Sensory testing of the distillates was done on a daily basis.

Good aseptic technique was followed in preparation of the yeast starter culture, and yeast growth and purity in the starter preparations and fermentations were monitored by microscopic observation and counting with a haemocytometer slide. High populations of large cells of the yeast (*S. cerevisiae*) were dominant in samples examined under the microscope but the staff were not trained to see the smaller sized, bacterial cells which were always present in such slide preparations. Consequently, bacterial populations within the production process at the distillery were not studied in any detail prior to this research project. Some culture plating for yeasts and bacteria were conducted but the rationale for this testing was not well understood. Samples of fermented molasses were diluted 10^3-10^4 fold before plating, consequently, species present at low populations would not be detected. There was a general assumption that because a pure yeast starter was used and that the molasses fermentation medium had been heated to 70-80°C for 40-60 min, the inoculated *S. cerevisiae* would be the only species present during fermentation.

Possible Sources of Lactic Acid Bacteria in the Rum Production Process

This was the first systematic ecological survey of an Australian rum distillery. Raw materials, in-process and fermentation samples were examined. The starter culture, *Saccharomyces cerevisiae,* was the only yeast found during fermentation stages. Investigations showed that the predominant bacteria were lactic acid bacteria. Four species of lactic acid bacteria were repetitively found in the fermentation samples with final populations reaching approximately 10^7 CFU/mL. The source of where these bacteria enter the production process is critical in order to obtain a thorough understanding of the microbial ecology of the distillery.

Samples of various raw materials and intermediate production components were taken throughout the study, with sampling sites becoming more specific and specialised as potential bacterial contamination sites were eliminated. This

systematic sampling often coincided with new information obtained from production staff regarding the complex array of pipework found in the production areas of the distillery.

Figure 3.10 is a reproduction of the process outline given in Figure 3.1, showing sites where lactic acid bacteria were isolated and key heat exchange units.

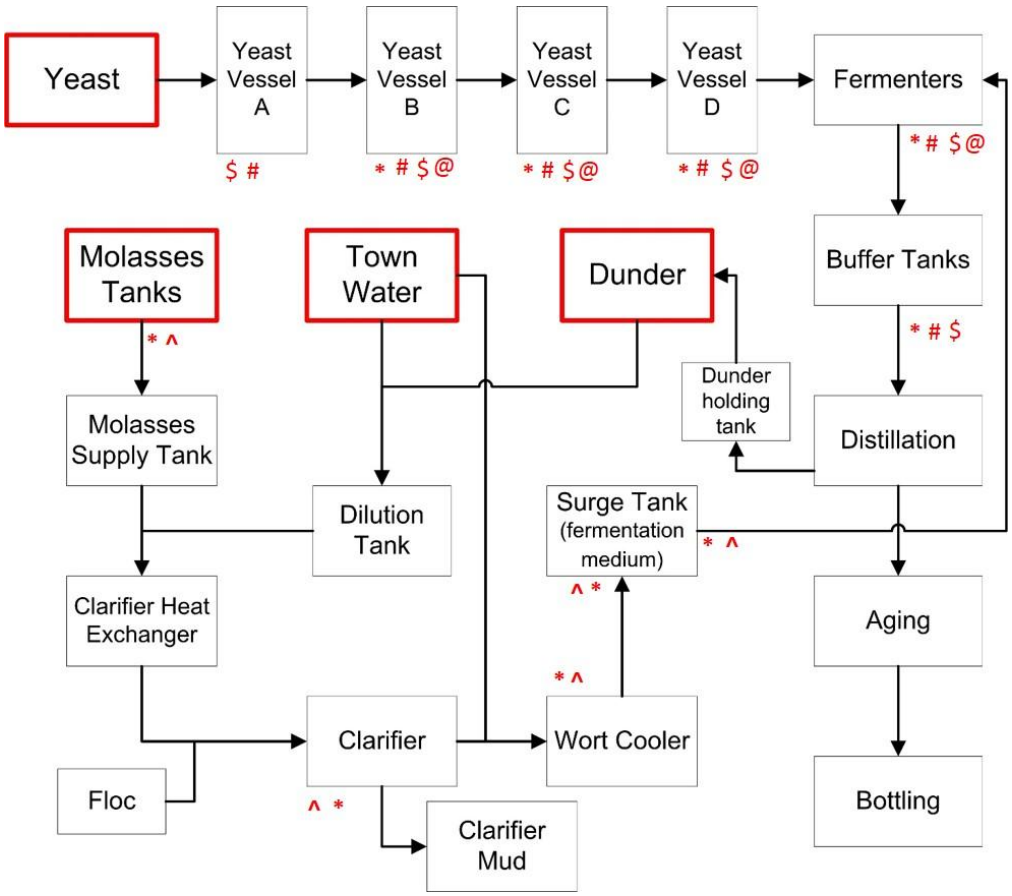


Figure 3.10 Flow diagram of production process showing sites where lactic acid bacteria were isolated and key heat exchangers,(*) *Lactobacillus* spp., ($) *L. plantarum, (#) L. fermentum*, (@) *L. brevis,* (^) all *Bacillus* species.

The unidentified *Lactobacillus* spp. was randomly found in samples of molasses, but at low populations and it carries through into the molasses fermentation medium and process. However, it is not the dominant species within the process. Aspects of its heat resistance will be discussed in Chapter 5. The main entry point

for *L. fermentum*, and *L. plantarum*, the two dominant bacteria of the process, (as well as *L. brevis* and the *Lactobacillus* spp.) is the scaled up yeast propagation stages where molasses fermentation medium was used. Sections of pipelines used to supply molasses fermentation medium to the propagation vessels were identified as those that escape effective CIP. It was not possible to dismantle and microbiologically examine these sections of pipeline during the course of this project. It is likely that biomasses of lactic acid bacteria have accumulated in sections of these lines and continually contaminate the molasses fermentation medium feeding into the yeast propagation vessels. Further research is needed to investigate this possibility.

Another likely source of contamination in the process arises from the dunder storage vessel, located between the feeding pipes from the distillation unit and the clarifier. Dunder is held in this tank on a transient basis as it is directly fed into the clarifier as needed to prepare the molasses fermentation medium. The temperature of dunder in this tank is in excess of 60°C. Samples of dunder taken from this tank throughout the course of the project were always at this temperature and, as expected, gave no culturable microorganisms. Dunder from this source is also used to give up its heat via heat exchangers to “cooling” waters that are continuously circulated throughout the production lines. It was revealed in the late stages of the project that these heat exchangers were not included in the CIP system and were only cleaned when they were seen to malfunction through accumulated materials and leakage into the cooling waters, as evidenced by a brown colouration of these waters. It was also revealed that the dunder storage tank is not within the CIP system since it is continuously hot, but there are times when production stops for one, two or more days when it cools down and develops off odours. When production restarts, this material is then fed into the system along with the microbial contaminants that have grown in the cooled dunder. In Chapter 5, it will be demonstrated that species of lactic acid bacteria develop in this dunder on cooling and storage. These contaminants will then be distributed throughout the production system through the heat exchangers that are not systematically maintained and cleaned. In this way, it may be concluded that lactic acid bacteria

become indigenous residential microflora of the production process, having the physiological ability to survive and grow in the environment that molasses presents. Such colonization is likely to occur in those sections of the process that escape effective CIP. Because of production pressures and timing constraints, it was not possible to undertake specific microbiological analyses of the dunder storage tank and associated heat exchangers to substantiate the above conclusion and this remains a direction for further research.

Conclusion

Microbiological surveys of the Bundaberg distillery over the period 2006-2010 showed that the inoculated yeast *S. cerevisiae* was responsible for the alcoholic fermentation of molasses for the production of rum.

Lactic acid bacteria were consistently found as major contributors to the fermentation process and originated as indigenous contaminants within the operation. Most likely sources were cross contamination from fermented molasses medium to raw materials that were being prepared for new fermentations. This in all likelihood arose due to a combination of factors such as under maintained equipment cleans and sporadic plant shutdowns causing temperature variations and unintentional lag times critical for bacterial growth.

Because these bacteria were present at quantitatively significant populations and likely to impact on product quality and process efficiency further research is needed to better understand their contribution to the process. Chapter 4 of this thesis will examine the bacterial ecology at the end of fermentation in more detail by studying the ecology of the buffer tanks. Chapter 5 will investigate the microbiological and chemical characteristics of the raw material, dunder and Chapter 6 will explore the impact of key bacterial species on the efficiency of yeast fermentation in a controlled environment and how this may affect production efficiency and flavour development.

CHAPTER 4

OCCURRENCE OF BACTERIA IN THE FERMENTATION OF MOLASSES FOR RUM PRODUCTION

4.1 INTRODUCTION

Alcoholic fermentation of molasses is a main operation in the production of rum. Yeasts are the primary microorganisms of this fermentation. There are numerous reports in the literature that bacteria may contribute to this fermentation (Allan, 1906; Hall *et al,* 1935; Arroyo, 1945a; Ganou-Parfait *et al* 1987 &1989), but a systematic, ecological study of the contribution of these bacteria is lacking. In Chapter 3, it was shown that bacteria, principally lactic acid bacteria, were part of the ecology of molasses fermentation for rum production at the Bundaberg Distilling Company. During fermentation, their populations increased to maximum levels of approximately 10^6-10^8 CFU/mL. The ecological investigations reported in Chapter 3 focussed on the entire production chain and, consequently, were limited to a few sampling times. Additional studies are required to confirm that lactic acid bacteria and, possibly, other species of bacteria are a consistent part of the microbial ecology of molasses fermentation.

In rum distilleries, it is not uncommon to use several tanks for the fermentation of molasses. At the completion of fermentation, the contents of these tanks are mixed into one tank called a buffer tank, from which the fermented molasses is fed into the distillation process. Such tanks are a quality control mechanism to help ensure a more uniform composition of fermented molasses for distillation. Due to mixing of

the contents of several fermenters into the one vessel, these buffer tanks provide a good sampling window to determine the bacterial populations and species in the production system.

To study these bacteria in more detail and to gain a greater knowledge of their prevalence in rum production over an extended period of time, the bacterial populations and species in buffer tanks at the Bundaberg distillery were determined over a period of 18 months.

4.2 MATERIALS AND METHODS

As part of its quality assurance program, the Bundaberg distillery routinely monitors the buffer tanks, for their populations of yeasts and bacteria, for each batch of rum production. These analyses are conducted by trained staff in a dedicated microbiology laboratory. Bacterial populations are monitored by the pour plate method using two culture media (i) Wallerstein Differential Nutrient Agar with Supplement (WLS) (Oxoid) and (ii) Raka Ray agar (Oxoid), each supplemented to contain 10 µg/mL of cycloheximide (Sigma) to restrict yeast growth. After on-site inspection, review of these procedures and training of staff, it was concluded that the plated cultures obtained by this routine quality assurance monitoring would be suitable for more detailed enumeration, isolation and identification of the specific bacterial populations associated with the rum process. For some fermentations, the laboratory staff were requested to include additional analyses by culturing samples from buffer tanks on de Man, Rogosa and Sharpe agar (MRS) agar also supplemented to contain 10 µg/mL of cycloheximide (Sigma). Details of the procedures are given in the following sections.

4.2.1 Samples from Buffer Tanks

Samples (30 mL) of fermented molasses were aseptically collected from the sampling ports of the buffer tanks, taken to the quality assurance laboratory on site

at the Bundaberg distillery, stored at 5°C, and analysed for the presence of bacteria within 1 h of collection. Samples for this study were collected over a period of approximately 18 months during August 2006-March 2008.

4.2.2 Analysis of Bacteria

Samples were serially diluted in 9 mL of Ringers solution and 1 mL sub-samples examined in duplicate for the populations of viable bacteria by the pour plate method using WLS agar (Oxoid) and Raka Ray agar (Oxoid). As per standard operating procedures used at the Bundaberg distillery, only the 10^5 dilution was used to generate the recorded population. Some samples were also examined, in duplicate, using MRS agar (Oxoid). Plates were incubated aerobically at 35°C. After incubation, total colony counts were calculated and the plates were aseptically packed in a cool box with ice bricks and shipped by courier to the University of New South Wales, Sydney. They were received within 2 days and then stored at 5°C until further examination, generally, within 2 days. Colonies on the plates were examined for their morphology and cellular morphology, and counts made for each specific colony type.

Representative isolates (5 of each colony type - if possible - each shipment) were isolated and purified by streaking onto plates of MRS and WLS agar. Plates were incubated microaerophilically using candle jars at 30°C for 48 hours. Stock cultures on slopes were prepared from these plates and stored under glycerol at -20°C and -80°C.

On several occasions samples from buffer tanks were analysed for the presence of *Propionibacterium* and *Zymomonas* species by culture on Sodium Lactate Agar and Universal Beer Agar respectively, as described in Section 3.2.2.2 Chapter 3. Some samples were also analysed for the presence of *Clostridium* species using duplicate pour plates of Differential Reinforced Clostridial Agar (RCA) (Difco). All three of these media were incubated anaerobically at 30°C for 3-7 days

(*Propionibacterium* and *Zymomonas* species) or 2-10 days (*Clostridium* species) and plates were checked for growth every 48 h. Anaerobic conditions were obtained using AnaeroGen© (Oxoid) in appropriate sealed containers.

4.2.2.1 Identification of Bacterial Isolates

Bacteria were identified by a combination of phenotypic methods and sequencing of the 16 S ribosomal DNA according to the methods described in Section 3.2.2 of Chapter 3. A minimum of 10 isolates of each species were isolated from plates of each medium type (WLS, Raka Ray and MRS) at different stages during the sampling period and their identities confirmed by rDNA sequencing.

4.3 RESULTS

Selection of isolation media

As mentioned already, the Quality Assurance Laboratory at the Bundaberg Distilling Company use WLS and Raka Ray media for the routine monitoring of bacteria in fermentation samples taken from buffer tanks. As part of this project, MRS agar was used as an additional medium for this purpose. Colony development on these media was slow and small for some species (e.g. the *Lactobacillus* spp.). In an attempt to improve the growth of these isolates, their growth on other media was examined at the outset of the project. These media included Plate Count Agar (PCA) (Oxoid) into which either 5% sucrose, 5% molasses or 5% dunder had been incorporated. However, trials with these media did not improve the growth of the bacteria on the plates or lead to the isolation of additional species.

4.3.1 Populations of Bacteria in Rum Fermentations

Throughout the study, a total of 66 sampling dates were examined. Table 4.1 shows the total populations as enumerated on either WLS, Raka Ray or MRS media. The first 44 of these samples were cultured on both WLS and Raka Ray

media. A further 22 samples were simultaneously plated on MRS agar as an additional check on media performance.

All of the sampled dates had populations of bacteria exceeding 10^6 CFU/ mL (on at least one medium), with many samples (59%) exceeding 10^7 CFU/mL and several (14%) exceeding 10^8 CFU/mL. These high populations were obtained on all three media, with no one medium giving consistently higher or lower populations for the same sample. The screening method, undertaken by the Bundaberg laboratory staff, had a limit of detection of $<5 \times 10^4$ CFU/mL. No consistent trends in population variations were observed in the samples taken over the 18 month time frame.

Table 4.1 Populations of bacteria (CFU/mL) in rum fermentation buffer tanks as determined by culture on WLS, Raka Ray and MRS agar plates

Sample	WLS	Raka Ray	MRS	Sample	WLS	Raka Ray	MRS
1	3.0×10^6	2.3×10^6	-[a]	*34*	1.4×10^8	2.6×10^7	-[a]
2	5.1×10^6	3.8×10^6	-[a]	*35*	4.6×10^8	1.0×10^7	-[a]
3	$< 5.0 \times 10^4$	4.5×10^6	-[a]	*36*	8.0×10^6	9.0×10^6	-[a]
4	7.3×10^6	6.5×10^6	-[a]	*37*	2.2×10^7	7.0×10^6	-[a]
5	1.8×10^8	2.5×10^6	-[a]	*38*	9.0×10^6	2.5×10^6	-[a]
6	$< 5.0 \times 10^4$	2.9×10^6	-[a]	*39*	4.2×10^7	4.9×10^7	-[a]
7	5.0×10^5	1.1×10^7	-[a]	*40*	$< 5.0 \times 10^4$	1.9×10^7	-[a]
8	1.5×10^6	$< 5.0 \times 10^4$	-[a]	*41*	$< 5.0 \times 10^4$	5.5×10^6	-[a]
9	$< 5.0 \times 10^4$	1.0×10^6	-[a]	*42*	$< 5.0 \times 10^4$	4.0×10^6	-[a]
10	$< 5.0 \times 10^4$	1.5×10^6	-[a]	*43*	2.4×10^7	$< 5.0 \times 10^4$	-[a]
11	1.3×10^7	1.5×10^6	-[a]	*44*	4.0×10^6	$< 5.0 \times 10^4$	-[a]
12	3.0×10^6	7.5×10^6	-[a]	*45*	4.5×10^6	2.1×10^7	4.5×10^6
13	4.0×10^6	3.5×10^6	-[a]	*46*	$< 5.0 \times 10^4$	1.8×10^7	1.5×10^6
14	$< 5.0 \times 10^4$	5.0×10^6	-[a]	*47*	2.7×10^7	3.0×10^6	1.4×10^7
15	$< 5.0 \times 10^4$	3.0×10^6	-[a]	*48*	1.0×10^8	1.0×10^6	3.0×10^6
16	$< 5.0 \times 10^4$	6.1×10^7	-[a]	*49*	5.6×10^7	1.9×10^7	3.2×10^7
17	4.5×10^6	$< 5.0 \times 10^4$	-[a]	*50*	1.0×10^6	1.2×10^7	1.0×10^6
18	1.5×10^7	$< 5.0 \times 10^4$	-[a]	*51*	3.0×10^6	1.8×10^7	1.0×10^5
19	3.5×10^6	$< 5.0 \times 10^4$	-[a]	*52*	3.5×10^6	2.9×10^7	1.0×10^6
20	2.2×10^7	$< 5.0 \times 10^4$	-[a]	*53*	7.5×10^6	4.7×10^7	3.5×10^6
21	5.0×10^5	$< 5.0 \times 10^4$	-[a]	*54*	3.6×10^7	1.1×10^7	3.0×10^6
22	3.0×10^7	$< 5.0 \times 10^4$	-[a]	*55*	3.2×10^7	4.0×10^6	3.0×10^6
23	3.5×10^6	$< 5.0 \times 10^4$	-[a]	*56*	4.0×10^6	$< 5.0 \times 10^4$	1.0×10^6
24	8.1×10^7	3.4×10^7	-[a]	*57*	8.4×10^7	3.3×10^7	8.0×10^6
25	1.1×10^8	3.0×10^6	-[a]	*58*	1.1×10^8	1.9×10^7	6.0×10^6
26	$< 5.0 \times 10^4$	3.2×10^7	-[a]	*59*	1.5×10^6	1.7×10^7	1.0×10^6
27	1.0×10^6	$< 5.0 \times 10^4$	-[a]	*60*	3.0×10^6	4.5×10^6	1.5×10^6
28	$< 5.0 \times 10^4$	2.5×10^7	-[a]	*61*	$< 5.0 \times 10^4$	$< 5.0 \times 10^4$	3.8×10^8
29	$< 5.0 \times 10^4$	1.4×10^7	-[a]	*62*	$< 5.0 \times 10^4$	$< 5.0 \times 10^4$	$>3.0 \times 10^8$
30	4.0×10^6	4.5×10^6	-[a]	*63*	$< 5.0 \times 10^4$	$< 5.0 \times 10^4$	1.0×10^6
31	1.5×10^7	$< 5.0 \times 10^4$	-[a]	*64*	2.0×10^6	2.1×10^7	5.5×10^6
32	$< 5.0 \times 10^4$	6.5×10^6	-[a]	*65*	1.0×10^7	1.2×10^7	1.5×10^6
33	$< 5.0 \times 10^4$	1.9×10^7	-[a]	*66*	1.3×10^8	2.2×10^7	9.5×10^6

[a] These samples were not tested on MRS agar

The results given are the mean counts of duplicate analyses.

4.3.2 Bacterial Species associated with Rum Fermentations

The colonies that developed on the WLS, Raka Ray and MRS isolation plates were consistently Gram positive, catalase negative rods and were considered as presumptive lactic acid bacteria. The colonial and cellular morphologies of the isolates, on these media, were similar to those described in Chapter 3. Four different morphological types were consistently present and were identified as *L. plantarum*, *L. fermentum*, *L brevis* and an isolate identified as an “unculturable

Lactobacillus" also described in Chapter 3. The "unculturable *Lactobacillus"* was a rod type isolate that was not precisely identified by sequence data but is presumptively described here as *Lactobacillus* spp. On some sporadic occasions (about twice over the 18 month survey period), colonies that did not match any of these four species were also noted on the isolation plates. The populations of these colonies (<10^5 CFU/mL) were less than those of the four main species found and, consequently, were not studied any further.

Lactobacillus fermentum was the most frequently occurring species and was isolated from 54 of the 66 samples (Table 4.2). *Lactobacillus plantarum* was the next most frequently isolated species, being present in 42 samples, followed by *Lactobacillus brevis* (27 samples) and *Lactobacillus* spp. (27 samples). Not all species occurred in the one sample at the same time but two to three species were generally present. There were only 13 occasions during the sampling period where only one species was present. Table 4.2 shows the population range for the different species. While most samples gave species in the population range 10^5-10^6 CFU/mL, some had *L. fermentum* and *L. plantarum* at population levels of 10^7-10^8 CFU/mL.

Table 4.2 Prevalence of species of lactic acid bacteria in rum fermentations

Species	**Population range (CFU/mL)**			**Total occurrence**
	10^5-10^6	**10^6-10^7**	**10^7-10^8**	
L. fermentum	33	18	3	54 (0.82)
L. plantarum	30	9	3	42 (0.64)
L. brevis	25	2	0	27 (0.41)
Lactobacillus spp.	22	5	0	27 (0.41)

From observations of the colony types recovered on each of the three isolation media, some differences were noted in the frequency of occurrence for the four species. Table 4.3 shows the number of times each species was isolated on each

medium. *Lactobacillus plantarum, L. fermentum and L. brevis* and the *Lactobacillus* spp. always grew on at least two of the three media used. However, Raka Ray agar had a higher frequency of recovery of *L. plantarum, L. brevis* and the *Lactobacillus* spp. *Lactobacillus fermentum* was more frequently recovered on WLS medium. *Lactobacillus fermentum* and *L. brevis* were not found in any samples plated onto MRS agar, although they could be isolated from these same samples when plated onto either Raka Ray or WLS media. *Lactobacillus* spp. and *L. plantarum* were the only two species isolated on all three media.

Table 4.3. Frequency of isolation of different species of lactic acid bacteria from rum fermentations on three different media

Medium	*Number of times growth occurred on medium*	*L. plantarum*	*L. fermentum*	*L. brevis*	*Lactobacillus* spp.
Raka Ray	50	33/50 (0.66)	24/50 (0.48)	30/50 (0.60)	18/50 (0.36)
WLS	47	15/47 (0.32)	45/47 (0.96)	2/47 (0.04)	3/47 (0.06)
MRS	22	12/22 (0.55)	nd	nd	10/22 (0.45)

(nd) not detected (species were not detected in populations above the limit of detection)
Note: Frequencies were calculated by dividing the number of times a species was isolated by the total number of samples with growth above the limit of detection for the test method.

Although analyses of the data obtained over the 2006-2008 sampling period enabled some broad conclusions to be made as shown in Tables 4.1 and 4.2, it was not possible to draw consistent conclusions about the populations for individual species. These populations fluctuated randomly from one sample to the next. This behaviour is illustrated in Figure 4.1 which shows the populations for individual species in samples collected over four different months in 2007.These data also confirm the greater prevalence and higher populations of *L fermentum* and *L. plantarum* that were observed over the entire sampling period.

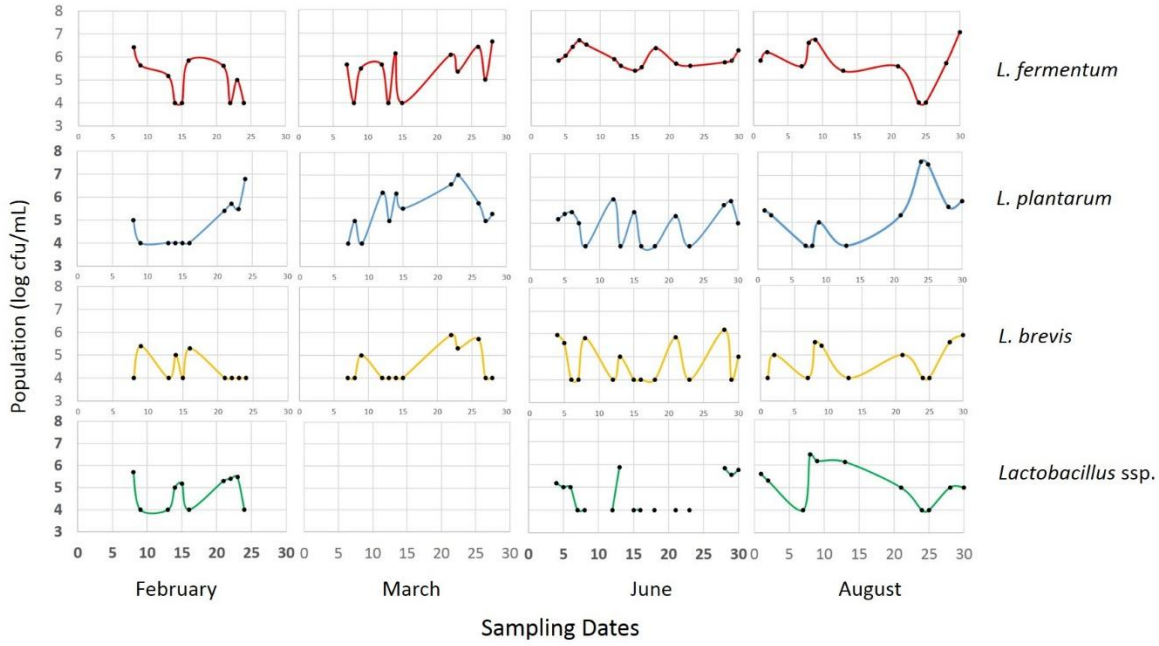


Figure 4.1 Populations of *Lactobacillus fermentum, Lactobacillus plantarum*, *Lactobacillus brevis* and *Lactobacillus* sp in samples of fermented molasses taken from buffer tanks over a four month period- February, March, June, August during 2007. Data represent the highest population observed on either of the three media used to analyse the sample.

Propionibacterium and *Zymomonas*

Buffer tank samples are not routinely tested for the specific presence of *Propionibacterium* and *Zymomonas* species. However, on five random occasions throughout the sampling period, samples of fermented molasses from the buffer tanks were analysed for the presence of these bacteria by culture on Sodium Lactate Agar and Universal Beer Agar, respectively as described in Chapter 3 (Section 3.2.2.2). None of the samples gave detection (< 50 CFU/mL) of these bacterial species.

Clostridium

Buffer tank samples are not routinely tested for the specific presence of *Clostridium* species. However, similarly to *Propionibaciterium* and *Zymomonas* species, five random occasions throughout the sampling period, samples of

fermented molasses from the buffer tanks were analysed for the presence of these bacteria by culture on Differential Reinforced Clostridial Agar (RCA) as described in Chapter 3 (Section 3.2.2.2). None of the samples gave detection (< 1 CFU/mL) of these bacterial species.

4.4 DISCUSSION

As described in Chapter 3, rum fermentations at the Bundaberg distillery are conducted in a battery of different fermenters, the use of which varies according to the volume of product required by marketing forces and other operational factors. Fermentation in each vessel usually lasts for about 24-36 h, after which the contents are discharged into one of two holding tanks, called buffer tanks. The fully fermented molasses in the buffer tanks is then continuously fed into the distillation units. As mentioned previously, this preliminary mixing or blending process also brings some uniformity or consistency into the product going to distillation.
As a consequence, the buffer tanks should provide a good overall picture of the microbial ecology of the fermentation process. Theoretically, microorganisms present in the buffer tanks should be the same as those present towards the end of fermentation in the individual fermentation vessels. Therefore, long term analyses of the microorganisms in the buffer tanks should provide a good, representative picture of the microbial ecology of the total operation, and was the focus of the investigations reported in this Chapter. In previous analyses, no variation was observed in the yeasts associated with the rum fermentations, this being the *S. cerevisiae* starter culture that was consistently used by the Bundaberg distillery. Therefore, analyses were confined to examination of the bacteria.

In designing the experimental protocol for this longitudinal study, several technical limitations, which could impact on the reliability of the data obtained, were recognized at the outset. These were:

- that the sampling, preparation of microbiological media, and cultural analyses would be conducted by technical staff at the Bundaberg distillery as part of their day to day work tasks, and that the reliability of their

contribution might be affected by unforeseen variables (e.g. pressures of day to day operation, staff absenteeism, changes within staff roles etc.)

- transportation of the cultured organisms on plates of isolation media from the Bundaberg distillery to the laboratories at the University of New South Wales (time delay and conditions of transport).

Although these variables could not be fully controlled, their impacts were minimized by visits to the Bundaberg distillery to train and ensure that staff were competent in the aseptic collection of samples, taking representative samples, preparation of microbiological media, plating and culturing of samples on the appropriate media, and packaging of cultured samples for transport. There were several samples in Table 4.1 where no bacteria were detected on one plating medium (< $50x10^4$ CFU/mL) but the same sample gave greater than 10^6 CFU/mL when plated on another medium. Such gross discrepancies are most likely due to analytical error within the laboratory, rather than an effect of plating medium composition.

On receipt at the University of New South Wales, samples were carefully checked for condition and any possible contamination, and were used within 24 hr. Samples that did not arrive within 2 days or were visibly damaged or contaminated were discarded. Moreover, to minimize any such influences, a large number of samples was taken and examined, this being some 66 samples that gave approximately 320 primary isolation plates from which cultures were isolated and examined.

Four species of bacteria were consistently isolated over an 18 month period. These were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus* spp. These were the same species that were found in the survey studies reported in Chapter 3, but within a very short time frame. The populations found for these bacteria ranged between < 5.0×10^4 CFU/mL and 3.8×10^8 CFU/mL and these data are also similar to those reported in Chapter 3. It is relevant to note that the culture technique used in this Chapter was based on pour plating while that reported in Chapter 3 used spread plating. Both approaches gave

similar ecological data with respect to the species and populations obtained, thereby reinforcing the reliability of the findings.

Reliability of the ecological data obtained was strengthened by the use of three media to isolate and culture the lactic acid bacteria. Although Raka Ray medium had the greatest number of isolations of lactic acid bacteria from the samples, and all four species could be isolated from this medium, it was less efficient at isolating *L. fermentum* than WLS medium. However, WLS was not particularly efficient at isolating *L. brevis* or the *Lactobacillus* spp., which was best isolated on MRS agar (Table 4.3). Conversely, MRS agar did not give reliable isolation of *L. fermentum* or *L. brevis*. There are numerous microbiological media available for the isolation and enumeration of lactic acid bacteria. This is due to the diversity of physiological properties exhibited by this group and, consequently, their presence in a large variety of matrices (Stamer, 1979; Carr *et al,* 2002), MRS agar is widely used for the isolation of lactic acid bacteria from many environmental/food samples (Stamer, 1979; Carr *et al,* 2002; Taskila *et al,* 2010), but, based on the data obtained in this study, it would not be appropriate as a sole medium for the analyses of molasses rum fermentations, and would lead to an underestimation of the ecology. The data of this Chapter clearly demonstrate the importance of using at least two, if not three, different isolation media in order to obtain reliable ecological data about the contribution of lactic acid bacteria to molasses rum fermentations. Media used for the isolation of LAB from rum fermentations have not been found in previous literature. . However, WL and Raka Ray media have been used for the isolation of these bacteria from cachaça fermentations ((Schwan, 2001; Badotti, 2010) and MRS based media have been used for their isolation from whisky fermentations (van Beek, 2002).
Similar to the data of Chapter 3, *Zymomonas, Propionibacterium* and *Clostridium* species were not detected in samples from the buffer tanks.

Although lactic acid bacteria were consistently associated with the rum fermentations at significant populations of 10^5 - 10^6 CFU/mL or more, there were

notable fluctuations in the presence and populations of individual species (Fig 4.1). The unidentifiable *Lactobacillus* spp. was not isolated from any sample during March 2007. However, it might have been present at lower populations, namely, less than the detection limit ($5.0x10^4$ CFU/mL) of the plating method used by the laboratory at the Bundaberg distillery. Such variations with the populations of the lactic acid bacteria are likely to affect the consistency of product quality and will need to be managed in quality assurance programs. As mentioned in Chapter 3, the different species of lactic acid bacteria have probably established themselves as part of an indigenous, residential microflora within the network of production facilities and pipelines. The extent and consistency of this colonisation will be determined by the frequency and effectiveness of cleaning and sanitation procedures and interruptions to the process that are likely to affect the growth and survival of these bacteria in the dunder holding tanks. Further research is recommended to confirm these possibilities.

As discussed in Chapter 3, the ecological data obtained are based on standard cultural techniques and further research using molecular based culture independent methods is recommended.

Conclusion

It can be concluded from the data of this Chapter, that lactic acid bacteria are a consistent component of the microbial ecology of molasses fermentation at the Bundaberg distillery. As discussed in Chapter 3, they originate as indigenous contaminants, having established themselves at locations in the process pipelines over many years. It is highly suspected that irregularities in the dunder management procedures gives rise to their presence after which they are inadvertently distributed to other locations within the process chain where residential colonisation has occurred. More detailed investigation of the microbiology of dunder will be presented in Chapter 5. The populations of lactic acid bacteria at the completion of fermentation are consistently high (approximately 10^6-10^7 CFU/mL) and, therefore, quantitatively significant in terms of their

metabolic contributions. Consequently, it is likely that they will affect the efficiency of the fermentation process and rum quality. Aspects of these influences will be investigated in Chapter 6.

CHAPTER 5

THE MICROBIAL ECOLOGY AND COMPOSITION OF DUNDER USED IN RUM PRODUCTION

5.1 INTRODUCTION

Rum is a distilled alcoholic beverage obtained from the fermentation of sugar cane molasses or sugar cane juice. Generally, production consists of preparation of the molasses or sugar cane juice, microbiological fermentation, distilling the ferment, maturation of the distillate, packaging of the product, and sale (Kampen, 1975; Lehtonen & Suomalainen, 1977; Bluhm, 1983; Fahrasmane & Ganou-Parfait, 1998; Nicol, 2003). Historically, dunder has been an ingredient of the process, and continues to be used in modern rum production (l'Anson, 1971; Kampen, 1975; Bluhm, 1983). Dunder is most often described as the residue in the still after distillation of the fermented molasses (Kampen, 1975). Dunder is taken from the Spanish word *"redundar*", meaning overflow (Martini, 2009). It is usually considered as a waste material and discarded into external pits or tanks where it is stored for collection as a feed for animals or as a fertilizer (Nicol, 2003). There seems to be no standard procedure for its use in rum production. It is mostly added to molasses fermentations at the time molasses is diluted with water. The amount added varies and can range from 0% to 50% but, mostly, it is added at about 0-10% (Olbrich, 1963; l'Anson, 1971; Fahrasmane & Ganou-Parfait, 1998). The reasons for its use are also varied and are described as: to lower molasses pH; provide an added source of nutrients for microorganisms for molasses fermentation; recycle part of the process water; and provide a source of wild yeasts and bacteria for molasses fermentation. Lowering the pH is thought to encourage the growth of *Schizosaccharomyces* yeasts in comparison to *Saccharomyces* yeasts during fermentation, and additional nutrients may encourage the growth of bacterial species that could favourably impact on rum flavours (Kampen, 1975;

Faharasmane & Ganou-Parfait, 1998). Frequently, dunder is stored before use and quickly undergoes a natural fermentation by yeasts and bacteria, the ecology of which is not well defined but, nevertheless, may impact on molasses fermentation and rum quality. Sometimes, this dunder has been used as a key source of microbial inocula for rum fermentations (Lehtonen & Suomalainen, 1977).

Despite the use of dunder in rum production and its potential impact on product quality and process efficiency, there is little published information on its microbiological and chemical properties. When examined under the microscope, it shows masses of yeast and bacterial cells, along with other particulate debris (Kampen, 1975). Theoretically, the microbial cells should be dead due to the high temperature of distillation. However, if the dunder is stored before use in rum fermentations, it is likely to become contaminated and support the growth of a specific microbiological flora. It is broadly mentioned throughout the literature that dunder is acidic (with high levels of butyric and acetic acid) and is enriched in nutrients such as amino acids, vitamins and peptides (Nicol, 2003). Dunder that is immediately recycled back into fermentations without being stored is generally considered to be sterile and is used in this way to reduce water consumption within the distillery and save on such costs.

The aim of this Chapter is to determine the microbial ecology and chemical composition of dunder and to investigate its effect on the growth of microorganisms during molasses fermentation.

5.2 MATERIALS AND METHODS

5.2.1 Dunder Samples

Samples (approximately 1000 mL) of dunder were collected on site from the Bundaberg Distilling Company over the period (March 2006 to September 2009). They were aseptically taken during commercial operation of the facility from sampling ports already built into the production lines. Dunder was allowed to flush through the sample ports prior to collection of the material for analysis, to ensure

representative samples were obtained. The dunder was not stored and came from a pipeline that was directly connected to the base of the distillation column. At the site of collection, its temperature was approximately 60°C. For chemical analysis, samples were frozen and stored at -20°C and thawed before examination. For microbiological analysis, they were stored at 5°C and examined within 24 hr. This dunder, collected and analysed on site is referred to as “fresh” dunder in the following sections.

After aseptic collection, some dunder samples were sent by courier to UNSW, Sydney. The time between collection of the samples and their receipt at UNSW was generally less than 7 days, and they were not refrigerated during this time, although they were packed with ice bricks to maintain as cool an environment as possible without freezing. On receipt, it was noted that the samples were undergoing microbial fermentation. These samples were stored at 4°C and then subjected to microbiological examination. This dunder, collected and transported to UNSW for further analysis is referred to as “stored” dunder in the following sections.

5.2.2 Microbiological Analysis of Dunder

Microbial flora of dunder was determined by (i) culture plating on agar media, (ii) enrichment culture followed by plating on agar media.

Samples (1.0 mL) of dunder were serially diluted in 0.1% Bacteriological Peptone Water, and 0.1 mL spread inoculated, in duplicate, onto plates of various agar media. Yeasts were analysed by plating onto Malt Extract Agar (MEA) and WL Nutrient agar (WL) supplemented to contain 100 µg/mL of oxytetracycline (Sigma) to restrict bacterial growth. The plates were incubated at 25°C for 48-72 h and checked for yeast colonies. Colonies with different morphologies were noted and separately counted to estimate their population. Representatives of the different colony types were isolated and purified by streak culture onto plates of MEA.

Bacteria were analysed by inoculation onto plates of Plate Count Agar (PCA), de Man, Rogosa, Sharpe Agar (MRS agar), Raka Ray (RR) and WL Nutrient Agar (WLS) each supplemented to contain 10 µg/mL of cycloheximide (Sigma) to restrict yeast growth. Plates were incubated at 30°C for 48 h, after which time colonies were counted. Predominant colony morphologies were noted, counted, and representative isolates were purified by streaking onto plates of MRS Agar (without cycloheximide).

To determine the presence of low populations of yeasts and bacteria in dunder, subsamples (1 mL) were subject to enrichment culture in test tubes of MRS and Malt Extract (ME) broth (50 mL) for 48 h at 30°C. The cultures were then tested for the presence of viable bacteria or yeasts by streak plating samples onto MRS agar for bacteria and MEA for yeasts.

All culture media were obtained from Oxoid (Basingstoke, UK) and prepared according to the manufacturer's instructions. Bacterial and yeast isolates from the above samples were stored at -80°C under 30% glycerol until used for identification.

5.2.3 Identification of Bacterial Isolates

Bacteria were identified by a combination of phenotypic methods and sequencing of the 16 S ribosomal DNA.

5.2.3.1 Phenotypic Tests

Phenotypic characterization included microscopic examination for cell morphology, Gram staining, and tests for oxidase and catalase reactions that were done according to standard procedures described in Smitbert and Krieg (1994). Isolates were then selected for identification using API CHL50 test strips (Biomerieux, Durham NC). Cultures were grown at 30°C for 24 hours on MRS Agar prior to collection and suspension of cell biomass in sterilised distilled water according to kit instructions. API kits were inoculated with the biomass and incubated at 30°C for 24 hours and observed for reactions. Kits were incubated for a further 24 hours,

reactions recorded and data processed using APIweb™ (http://apiweb.biomerieux.com) to give bacterial genus and species identification.

5.2.3.2 Extraction and Sequencing of ribosomal DNA

Bacterial DNA was extracted, amplified and sequenced according to the methods found in Chapter 3, Section 3.2.2.2.

The products were sequenced at the Ramaciotti Centre for Gene Function Analysis, UNSW, Australia. The resulting sequences underwent DNA similarity searches on NCBI Blast program using sequences retrieved from the Genebank Database (Karlin & Altschul, 1990).

5.2.4 Microscopic Examination

5.2.4.1 Dunder Samples

Samples of dunder were examined under normal light/ fluorescence microscopy to determine their general microbial load and composition. These samples were (1) dunder samples taken directly from the distillery, not stored and not undergoing any fermentation and (2) dunder samples that had been couriered to UNSW and were undergoing fermentation. Microbial cells in dunder were sedimented by centrifugation (2 minutes at 10 000g) washed twice by aspiration with 1 x PBS (phosphate buffered saline) and stained for 10 min with SYBR Green I Nucleic acid stain (Invitrogen) (1:1000 in PBS). Cells were centrifuged (2 minutes at 10 000g) and resuspended in 1 mL 1x PBS. Samples of the cell suspension were transferred to a slide and analysed using a BH 2 epi-fluorescence microscope 100X oil, objective (N.A 1.4) (Olympus, Japan), Excitation 488nm – Emission (Jin, X *et al.,* 1994). This work was done in conjunction with the Microscopy Unit, Department of Biological Sciences, Macquarie University, NSW, Australia.

5.2.4.2 Bacterial Isolates

Bacterial isolates were examined by microscopy according to the methods described in Chapter 3, Section 3.2.3.

5.2.5 Isolation of Bacteria from Stored and Heated Dunder

In principle, dunder samples taken after distillation should be sterile because of the time and temperature of the distillation process. This was a continuous feed process at 5 000 mL/ minute at 103°C. Dunder sampled and examined immediately after distillation showed no viable microorganisms (see Results). However, dunder samples aseptically collected and shipped to UNSW were consistently fermenting on arrival, suggesting the presence of some heat resistant microorganism(s) that recovered from heating stress and subsequently grew. Consequently, experiments were conducted to test for the presence of heat resistant species in these stored and fermenting samples. Samples (500 mL) of these fermenting dunders were transferred to a pre-sterilised glass bottle with loosened cap and heated on a hot plate with magnetic stirring at 100°C for 15 min. After heating, the bottle of dunder, covered with cap to prevent recontamination),was incubated at 25°C for 4 days Samples (10 mL) were taken immediately after heating and thereafter at 2 and 4 days during incubation and spread plated (0.1 mL) onto MRS agar and MEA to check for the presence of viable bacteria and yeasts. Plates were incubated at 30°C for 48 hours, after which time any colonies present were counted and isolated onto MRS agar. Isolates were purified by restreaking and stored as described previously, and identified to genus and species by phenotypic and nucleic acid sequencing as described in Section 5.2.2.2.

5.2.5.1 Characterisation of Bacterial Isolates from Heated Dunder

Isolates from Section 5.2.2 were subjected to further characterization because they were not conclusively identified by the phenotypic and DNA sequencing

determination described in section 5.2.3.2. Further characterization included growth at different temperatures and pH and ethanol tolerance.

Stock cultures were grown in MRS broth for 24-48 hours prior to inoculation into MRS broth prepared according to the relevant test. Initial population was approximately 10^7 CFU/mL.

5.2.5.2 Temperature Tolerance

Growth at different temperatures was conducted in MRS broth (100 mL) in 120 mL bottles. The medium was inoculated with 100 µL of stock culture and incubated at either 20°C, 25°C, 30°C, 40°C, 50°C or 60°C in triplicate. Growth was monitored by measurement of increase in optical density (OD) (600 nm) (UV1201, Shimadzu) due to biomass formation. Samples of culture were taken every 8 h for measurement. One bottle of medium (blank) was also incubated at each temperature to ensure any discolouration of the medium due to temperature would be taken into account. Experiments were done in triplicate on two separate occasions and average values are reported.

5.2.5.3 Ethanol Tolerance

Ethanol tolerance was determined by measuring growth in MRS broth (final volume 100mL) to which ethanol had been added after autoclaving, to give final concentrations of 0%, 1%, 2.5%, 5%, 7.5% and 10% v/v of ethanol. Media were inoculated with 100 µL of stock culture and incubated at 30°C. Growth was monitored by OD measurement as described previously, and experiments done in triplicate on two separate occasions and average values are reported.

5.2.6 Effect of Dunder on the Growth of Yeasts and Bacteria in Molasses

Molasses and dunder were obtained from the Bundaberg Distilling Company. The molasses was diluted with water to give the concentration normally used in rum fermentation, namely, 30°Brix or 15% fermentable sugars. Part of the water was replaced with dunder to give diluted molasses containing 10%, 25% and 50% v/v of dunder, as well as the control, 0% dunder, where no dunder was added. The molasses medium was adjusted to pH 5.5 by addition of 5M HCl. The medium was dispensed as 1000 mL volumes in 1L Schott bottles and sterilized by autoclaving.

Fermentations of the molasses media were conducted with *Saccharomyces cerevisiae, Lactobacillus plantarum, Lactobacillus fermentum* and *Lactobacillus* spp. (Chapter 3 &4). Inoculum cultures of *S. cerevisiae* were prepared from fresh slants of MEA and cultured in MEB at 30°C for 24-48 h. This culture (5 mL) was inoculated into 100 mL of molasses medium and incubated at 30°C for 24-36 h. The resulting culture (2 mL) was used to inoculate molasses medium containing different concentrations of dunder. Inoculum cultures of each of the *Lactobacillus* species were similarly prepared except that they were pre-cultured in MRS broth.

The molasses cultures were incubated at 30°C for 48 h without shaking and samples (10 mL) were taken aseptically every 6 h until 48 h for measurements of yeast or bacterial growth by plate culture. Samples were serially diluted in 0.1% Bacteriological Peptone Water, and 0.1 mL spread inoculated, in duplicate, onto plates of MEA for yeasts and MRS agar for the *Lactobacillus* species. All plates were incubated at 30°C for 48 h and colonies were counted.

5.2.7 Chemical Composition of Dunder

Samples of dunder were analysed for pH, sugars, organic acids and free amino acids.

5.2.7.1 Sample preparation

Samples to be analysed for sugars and organic acids were prepared according to a modified version of Ardhana and Fleet (2003). Twenty grams of sample (dunder) were diluted with 100 mL of distilled water. The homogenate was centrifuged (Beckman-Coulter, Fullerton, CA) at 4°C 20,000 x g for 20 min and the supernatant collected. The supernatant was filtered under vacuum through a 0.45µm non-sterilised filter membrane (Millipore).

5.2.7.2 pH

These analyses were done using an Activon© optical pH probe for pH on uncentrifuged samples, taken directly from the either the fresh or stored dunder sample. Analyses were done in duplicate and average values are reported.

5.2.7.3 Organic acids

The concentration of individual organic acids was determined by HPLC analysis. Chromatography instrumentation consisted of a pump (Waters 600), autoinjector (Waters 717, Autosampler PLUS), a PDA-UV detector (Waters 996 PDA) programmed to extract at a wavelength of 210 nm, a column heater and controller (Waters Temperature Control Module) and a 300 x 7.8 mm Aminex Ion Exclusion HPX-87H stainless steel column (BioRad, Richmond CA). This system was controlled by a computer running Waters Millenium ™ software and data recorded simultaneously. The column was eluted at 65°C using 0.08% H_3PO_4 (Ajax Chemicals, Sydney, Australia) at a flow rate of 0.5 mL/min. Stock solutions of acetic acid (UNIVAR), oxalic acid 99% (BDH), tartaric acid 99% (Sigma Aldrich), malic acid 99% (Sigma Aldrich), lactic acid 99% (BDH), propionic acid 99% (Sigma Aldrich), butyric acid (as 4-phenyl butyric acid, 99%) (Sigma Aldrich), succinic acid 99% (Cole Palmer) and citric acid 99% (Sigma Aldrich) were used to produce a mixed standard calibration curve to identify and quantify the concentration of each acid. The calibration curve consisted of three points at 1.5%, 1.0% and 0.5%. Individual vials of each acid were analysed to determine exact retention time

(location) in the chromatogram produced by the HPLC. Analyses were done in duplicate and average values are reported.

5.2.7.4 Sugars

The concentration of individual sugars was determined by HPLC analysis. Chromatography instrumentation consisted of a pump (Waters 600), autoinjector (Waters 717, Autosampler PLUS), a RI detector (Waters RI) coupled with a PDA-UV detector (Waters 996 PDA) programmed to extract at 210 nm, a column heater and controller (Waters Temperature Control Module) and a 300 x 7.8 mm Aminex Ion Exclusion HPX-87H stainless steel column (BioRad, Richmond CA). This system was controlled by a computer running Waters Millenium ™ software and data recorded simultaneously. The column was eluted at 65°C using 0.005 M H_2SO_4 (Ajax Chemicals, Sydney, Australia) at a flow rate of 0.6 mL/min. Stock solutions of sucrose (Sigma Aldrich), d-fructose (Sigma Aldrich) and glucose (sigma Aldrich) were used to produce a mixed calibration curve (to identify and quantify the concentration of each sugar. The calibration curve consisted of three points at 60 mg/mL, 50mg/mL and 40mg/mL. Individual vials of each sugar were analysed to determine exact retention time (location) in the chromatogram produced by the HPLC. Analyses were done in duplicate and average values are reported.

Lactose was used as an internal standard to determine any loss through the sample preparation method. Five mL of the stock solution (32g/L) was added prior to sample preparation.

5.2.7.5 Amino acids

Dunder samples (fresh and stored) were sent to the Australian Proteome Analysis Facility (Macquarie University, North Ryde, NSW) for analysis of free amino acids. Samples (4 µL) were analysed, in duplicate, using the Waters AccQ-Tag Ultra chemistry with quantification by Waters ACQUITY UPLC. The analysis was facilitated using infrastructure provided by the Australian Government through the

National Collaborative Research Infrastructure Strategy (NCRIS). Analyses were done in duplicate and average values are reported.

5.2.7.6 Ethanol

Ethanol concentrations were determined from samples prepared as described in Section 5.2.6.1; however, vacuum filtration was not used. Aliquots (2 mL) of supernatant after initial centrifugation were subjected to a secondary centrifugation step (4°C, 15,000 x g for 10 min). Ethanol concentrations were determined in this supernatant using an enzymatic assay kit (r-biopharm AG, Germany). Analyses were done in duplicate and average values are reported.

5.2.7.7 Volatile Compounds

Volatile compounds were determined from samples prepared as described in Section 5.2.6.1, but vacuum filtration was not used. Aliquots of supernatant after initial centrifugation were subjected to a secondary centrifugation step (4°C, 15,000 x g for 10 min). Samples were then dispatched to The Australian Wine Research Institute under refrigerated temperature conditions. There they underwent in-house sample preparation as described below.

Samples were prepared in 2 dilutions 1/20 and 3/10 with buffer (10% potassium hydrogen tartrate, pH adjusted with tartaric acid to 4.5). Samples were prepared and analysed in a randomised order with a blank run every 10 samples.

The analysis was performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS1 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis was performed with Agilent G1701A Revision E.02.00 ChemStation software. The gas chromatograph was fitted with a 30 m x 0.18 mm Resteck Stabilwax – DA column (crossbond carbowax polyethylene glycol) of 0.18 µm film thickness that had a 5m x 0.18mm retention gap. Helium (Ultra High Purity) was used as the carrier gas with linear velocity 24.6cm/s, flow rate 0.78mL/min in constant flow mode. The oven temperature was

started at 33°C, held at this temperature for 4 min then increased to 60°C at 4°C/min, then heated at 8°C/min to 230°C and held at this temperature for 5 min.

The conditions of large volume headspace sampling used were as follows: the vial and its contents were heated to 40°C for 10 minutes with agitation (speed 750 rpm, on time 80 s, off time 1 s). A heated (55°C) 2.5 mL syringe penetrated the septum (27.0 mm) and removed 2.5 mL of headspace (fill speed 200 µL/s). The contents of the syringe were then injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA inlet liner (0.75 mm I.D., pre-conditioned in the GC inlet at 200°C for 1 hour and then ramped to 350°C to remove all contaminates before first injection).

The inlet conditions used were as follows: Prior to injection the inlet was cooled to 0°C with liquid nitrogen. While maintaining 0°C, the sample was introduced to the inlet at 25.0µL/s (penetration 22.0 mm) using split mode (split ratio 33:1, split flow 25.78 mL/min). Following capture of analytes on the Tenax liner the injector was heated to 330°C at 12°C/min (pressure 24.6).

The mass spectrometer conditions used were as follows: The mass spectrometer quadrupole temperature was set at 150°C, the source was set at 250°C and the transfer line was held at 280°C. Positive ion electron impact spectra at 70eV were recorded in selective ion monitoring (SIM) mode and Scan mode simultaneously (relative EM volts) with a solvent delay of 4.0 min.

All quantitative data processing was performed with Agilent G1701A Revision E.02.00 ChemStation software. Analyses were done in duplicate and average values are reported.

5.3 RESULTS

5.3.1 Yeast and Bacterial Populations in Dunder Samples

Five samples of dunder collected and analysed on site at the rum distillery on separate occasions gave no viable yeasts (< 5 CFU/mL) when plated onto MEA and WL agar and no viable bacteria (< 5 CFU/mL) when plated onto MRS, WLS, PCA and RR agars (Table 5.1). The same samples were also analysed by enrichment culture in ME broth and MRS broth and gave no viable yeasts or bacteria after enrichment (Table 5.1).

Table 5.1. Yeast and bacterial counts of dunder sourced directly from the rum distillery and analysed by direct plating and enrichment.

Sample	Media used	Dilutions Performed	Population (CFU/mL)
1	MEA, WL,	10^0, 10^{-1}, 10^{-2}	<5
	MRS, PCA	10^0, 10^{-1}, 10^{-2}	<5
2	MEA, WL	10^0, 10^{-1}	<5
	RR, MRS	10^0, 10^{-1}	<5
3	MEA, WL	10^0, 10^{-1}	<5
	RR, MRS, WLS	10^0, 10^{-1}	<5
4	MEA, WL	10^0, 10^{-1}	<5
	MRS, WLS, PCA	10^0, 10^{-1}	<5
5	MEA, WL	10^0, 10^{-1}	<5
	MRS, WLS, PCA	10^0, 10^{-1}	<5
4*	MEA, WL	10^0, 10^{-1}	<5
	MRS, WLS, PCA	10^0, 10^{-1}	<5
5*	MEA, WL	10^0, 10^{-1}	<5
	MRS, WLS, PCA	10^0, 10^{-1}	<5

Note: Samples 4* and 5* were subjected to enrichment in MRS or ME broth for 24 hours prior to plating onto agar.

When examined under the light microscope, these samples of dunder showed high concentrations of yeast cells, and lesser populations of small to long rod like bacterial cells (Figure 5.1). The staining with SYBR ® Green 1 dye confirmed the phase contrast results, with large amounts of yeast cells visible and smaller quantities of bacteria also present (Figure 5.1 c & e).

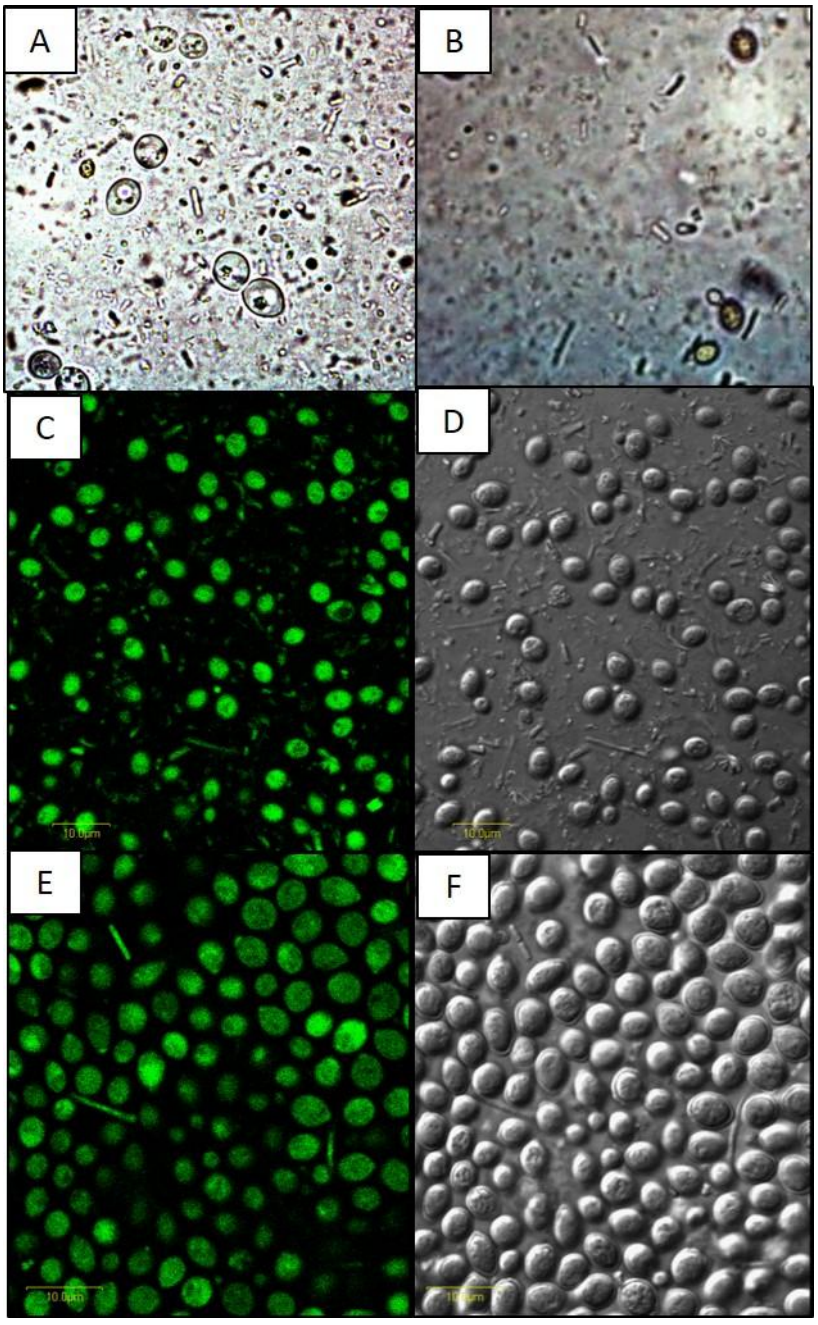


Figure 5.1 Micrographs of dunder examined under phase contrast and epifluorescence microscopy (magnification x 1000) (a) fresh dunder; (b) stored dunder; (c) stored dunder stained with SYBR green I dye and exposed to epifluorescence; (d) stored dunder without epifluorescence excitation; (e) fresh dunder stained with SYBR green I dye and exposed to epifluorescence; (f) fresh dunder without epifluorescence excitation

Dunder samples received after courier transport to UNSW were fermenting on arrival. The plastic bottles containing the dunder samples were visibly swollen and the dunder was gassy. Table 5.2 shows the results of microbiological analysis of

these samples. No yeasts (< 5 CFU/mL) were detected after culture on MEA and WL agar. However, all samples showed the presence of bacteria when plated onto MRS agar. Total populations ranged from 1.8 x 10^6 CFU/mL to 1.9 x 10^8 CFU/mL. Based on colony morphologies, at least two different species were present in these samples.

Table 5.2. Yeast and bacterial counts of dunder aseptically sampled from the rum distillery and examined after transport to UNSW-approximately 4-7 days.

Sample	Date	Bacteria (CFU/mL)		Yeast (CFU/mL)
		A	B	
1	04/12/06	3.4 x 10^8	<5.0 x 10^2	<5
2	29/01/07	8.9 x 10^7	<5.0 x 10^2	<5
3	28/03/07	1.8 x 10^6	1.2 x 10^5	<5
4	30/03/07	1.9 x 10^8	4.5 x 10^6	<5
5	16/05/07	5.2 x 10^7	1.5 x 10^6	<5
6	16/07/07	5.6 x 10^7	<5.0 x 10^2	<5
7	20/09/07	2.7 x 10^7	<5.0 x 10^2	<5
8	15/07/09[a]	2.0 x 10^6	2.3 x 10^3	<5
9	15/07/09[b]	2.7 x 10^6	2.8 x 10^3	<5
10	12/01/10	4.9 x 10^6	<5.0 x 10^2	<5

A- Cream colony 1-3 mm diameter, defined edge, circular

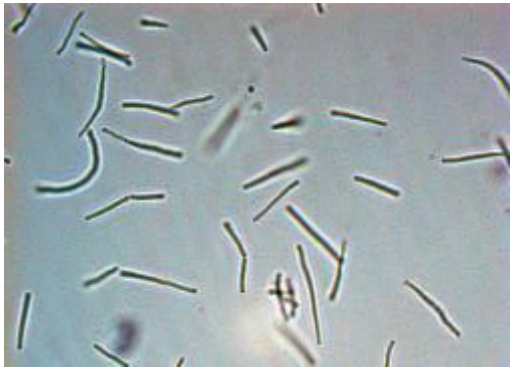
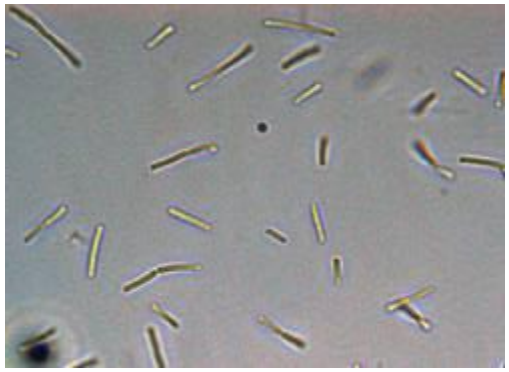
B- Pale brown irregular edge 1-2 mm diameter

[a] sample taken early (8:30am), [b] sample taken late (2pm)

5.3.2 Identification of Bacteria Isolated from Dunder

Dunder samples contained two main types of colonies of bacteria (Table 5.2). These were isolated and characterised. Table 5.3 shows the colonial and cellular morphologies of the two isolates. Both isolates were distinctive as long, thin rod-shaped cells. Both isolates tested positive for the Gram reaction and negative for the catalase and oxidase reactions. Based on these criteria, the isolates were tentatively classified within the genus of *Lactobacillus* for further testing. Sequencing of the rDNA of both of the isolates gave identical non-specific results with closest matches (~97-99%) to an “unculturable *Lactobacillus* species”. Phenotypic identification using API CHL50 test strips gave both isolates as *Lactobacillus acidophilus*.

Table 5.3. Morphology, rDNA sequencing and API identification of bacteria isolated from dunder

Morphological description	Plate culture	Light microscopy	Sequencing	API identification
Pale brown circular colony, 2-3 mm in diameter			Unculturable Lactobacillus (GQ082129.1) 99.0%	Lactobacillus acidophilus 92.7% (Good ID)
Cream, circular colony, 2-4 mm in diameter			Unculturable Lactobacillus (GQ082129.1) 98.0%	Lactobacillus acidophilus 92.7% (Good ID)

5.3.3 Isolation and Identification of Bacterial Species from Heated Dunder

It was noted that, after heating, dunder samples sometimes re-fermented, suggesting the presence of heat resistant organisms. To investigate this observation more thoroughly, dunder samples were heated to boiling as described in Section 5.2.3.1, allowed to cool and then sampled for microbiological analysis during subsequent storage at 25°C. Out of five different dunder samples treated in this way, two re-fermented, from which one bacterial species was isolated as shown in Figure 5.2.

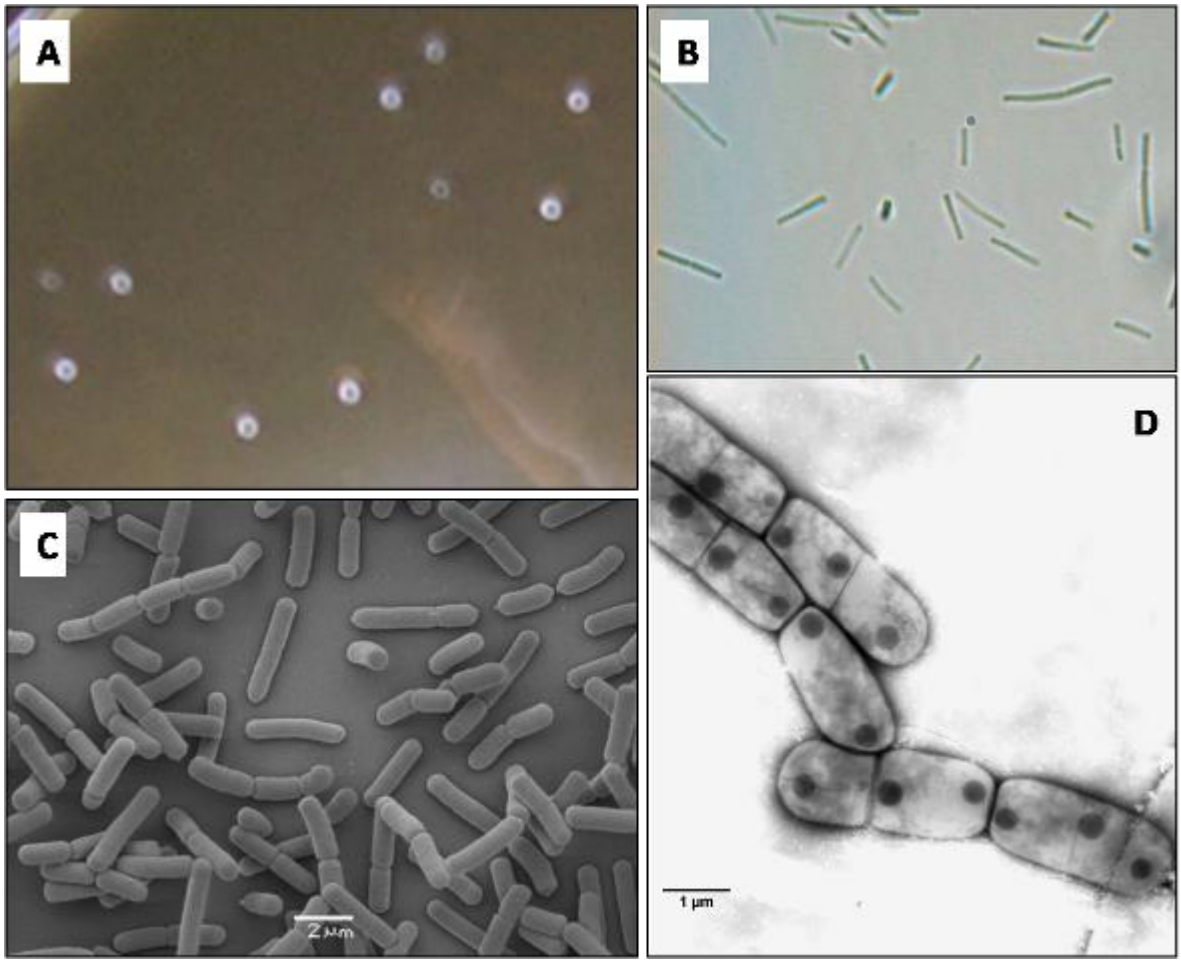


Figure 5.2. Bacterial isolate from dunder samples which had re-fermented after boiling. (A) Colony on MRS agar (B) Cells examined under light microscopy (x 1000, Leica) (C) Cells examined with Scanning Electron Microscopy and Transmission Electron Microscopy (D) (Electron Microscope images were taken at Macquarie University Microscopy Unit, NSW, Australia)

This organism had a pin-head sized, cream colony with dark centres on MRS agar and characteristic long thin rods under microscopic examination .The isolate was Gram positive, catalase negative and oxidase negative. It was tentatively assigned to the genus of *Lactobacillus* for further testing. rDNA sequencing of the isolates gave non-specific results with closest matches (~97-99%) to an “uncultureable” *Lactobacillus* species. Phenotypic identification API CHL50 test strips gave a closest match 92.7% to *Lactobacillus acidophilus*. Table 5.4 shows the API carbohydrate fermentation profile tested on four different isolates of the same colonial appearance in comparison to data obtained from the literature for *L. acidophilus*

Table 5.4 Carbohydrate fermentation reactions of dunder isolates as determined by API 50CHL analyses

Fermentation of::	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
Galactose	+	+	+	+	+	+	+	+	(+)	+	v
Glucose	+	+	+	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	v
N-acetyl glucosamine	+	+	+	+	+	+	+	+	(+)	+	(+)
Amygdalin	+	+	+	+	+	+	+	+	v	(-)	-
Arbutin	+	+	+	+	+	+	+	+	v	-	-
Aesculin	+	+	+	+	+	+	+	+	+	+	v
Salicin	+	+	+	+	+	+	+	+	+	v	v
Cellobiose	+	+	+	+	+	+	+	+	+	(+)	v
Maltose	-	-	-	-	-	-	-	-	(+)	+	(+)
Lactose	+	+	+	+	+	+	+	+	v	+	v
Starch	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	(+)	-	v
Raffinose	-	-	-	-	-	-	-	-	(-)	(+)	(-)
Amygdalin	-	-	-	-	-	-	-	-	(-)	(+)	-
Gentiobiose	+	+	+	+	+	+	+	+	+	+	-
D-tagatose	+	+	+	+	+	+	+	+	v	(-)	-

Note: Data in columns (1), (2), (3), (4) were results determined in this study (Chapter 5). Column (5) & (6) results obtained from isolates matching the same rDNA results during ecological survey performed in Chapter 3, (7) & (8) results obtained from isolates matching the same rDNA results during buffer tank survey performed in Chapter 4, (9), (10) and (11) were sourced from API 50 CHL literature regarding *L. acidophilus* (as per API CHL information brochure, Biomerieux, France). Characters are scored as + (85-100%), (+) 75-84% positive, v variable (26-74% positive), (-) 16-25% positive and – 0-15% positive.

Note: (1)-(8) results according to API CHL 50 (Biomerieux) were L. acidophilus 92.7% Good ID,

The morphology of the isolate as a rod shaped bacterium often in pairs or short chains was confirmed by scanning and transmission electron microscopy (Figure 5.2). This corresponds to the identification and classification of the “unculturable” *Lactobacillus* species isolated during the ecological survey performed at the Bundaberg distillery as described in Chapter 3 and one of the bacterial species isolated from the buffer tanks in Chapter 4.

5.3.4 Growth of the *Lactobacillus* spp. Isolate from Heated Dunder in the Presence of Ethanol and at Different Temperatures

Some growth experiments were undertaken to determine the tolerance of the isolate to ethanol and temperature conditions that occur in the distillery environment.

Figure 5.3 shows growth of the isolate in MRS broth adjusted to contain ethanol concentrations up to 10%. Growth was completely inhibited in the presence of 7.5% and 10% ethanol concentrations. Growth rate and growth yield were substantially decreased in the presence of 2.5% and 5% ethanol. In the case of 5% ethanol, the growth rate was approximately 50% that of the control. The addition of 1% ethanol, impacted slightly on growth of the bacterium, with final cell density being slightly less than that obtained in the absence of ethanol.

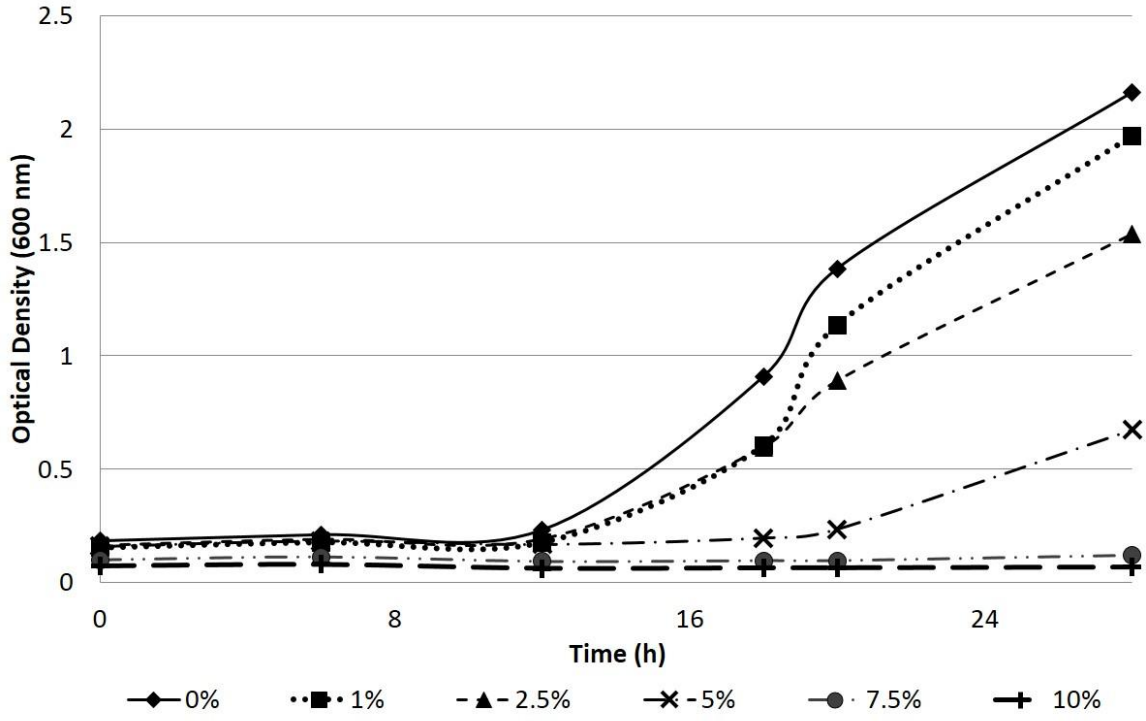


Figure 5.3 Effect of ethanol concentration on the growth of a *Lactobacillus* spp. isolated from heated dunder.

The effect of varying temperatures on the growth of *Lactobacillus* spp. is shown in Figure 5.4. Fastest growth occurred at 30°C followed by 40°C. Significant lag phases were obtained for growth at 20°C and 25°C, although by 24 h, final cell densities were similar to those found for growth at 30°C and 40°C. No growth was observed at 50°C or 60°C.

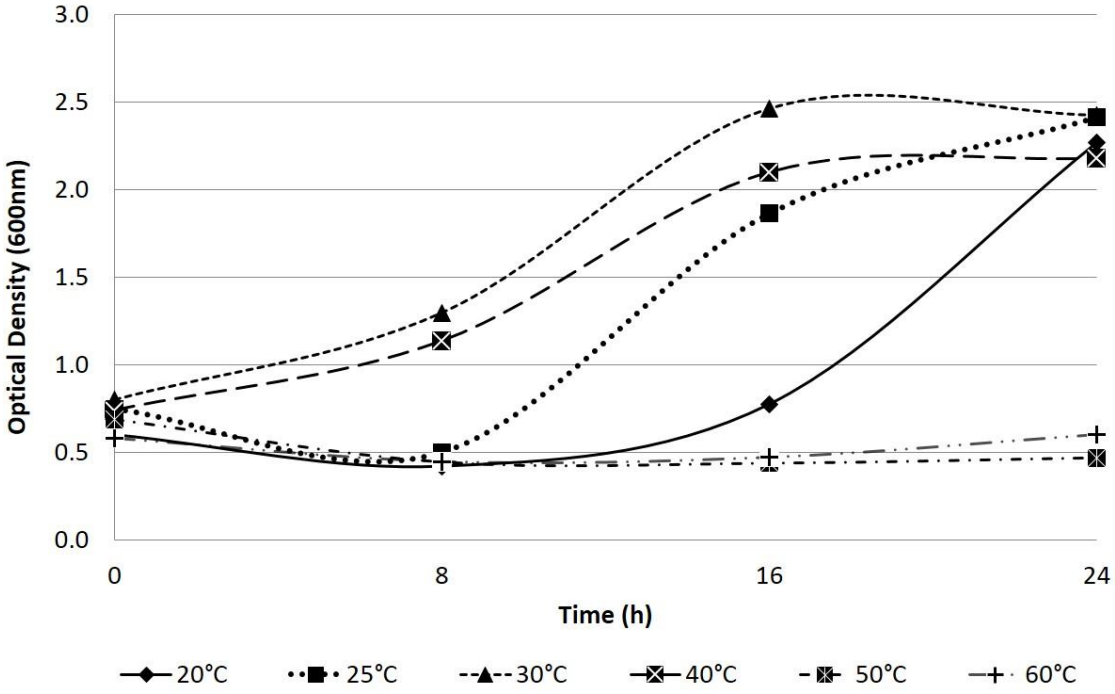


Figure 5.4 Effect of temperature on the growth of *Lactobacillus* spp. isolated from dunder.

The appearance of the culture medium during growth at 40°C was clearly different to that for growth at lower temperatures (Figure 5.5). At 40°C, the cell biomass tended to come out of a turbid suspension and clump at the bottom of the vessel (Figure 5.5 b), whereas the biomass was more uniformly suspended and turbid for growth at 30°C (Figure 5.5 a). Samples from these cultures also revealed different appearances when examined by light and electron microscopy. The bacterial cells at 40°C were more clumped and aggregated (Figure 5.5 d) than those grown at 30°C (Figure 5.5 c). This change in cell behaviour was also observed during electron microscopy imaging of the 30°C (Figure 5.5 e) and 40°C (Figure 5.5 f) samples.

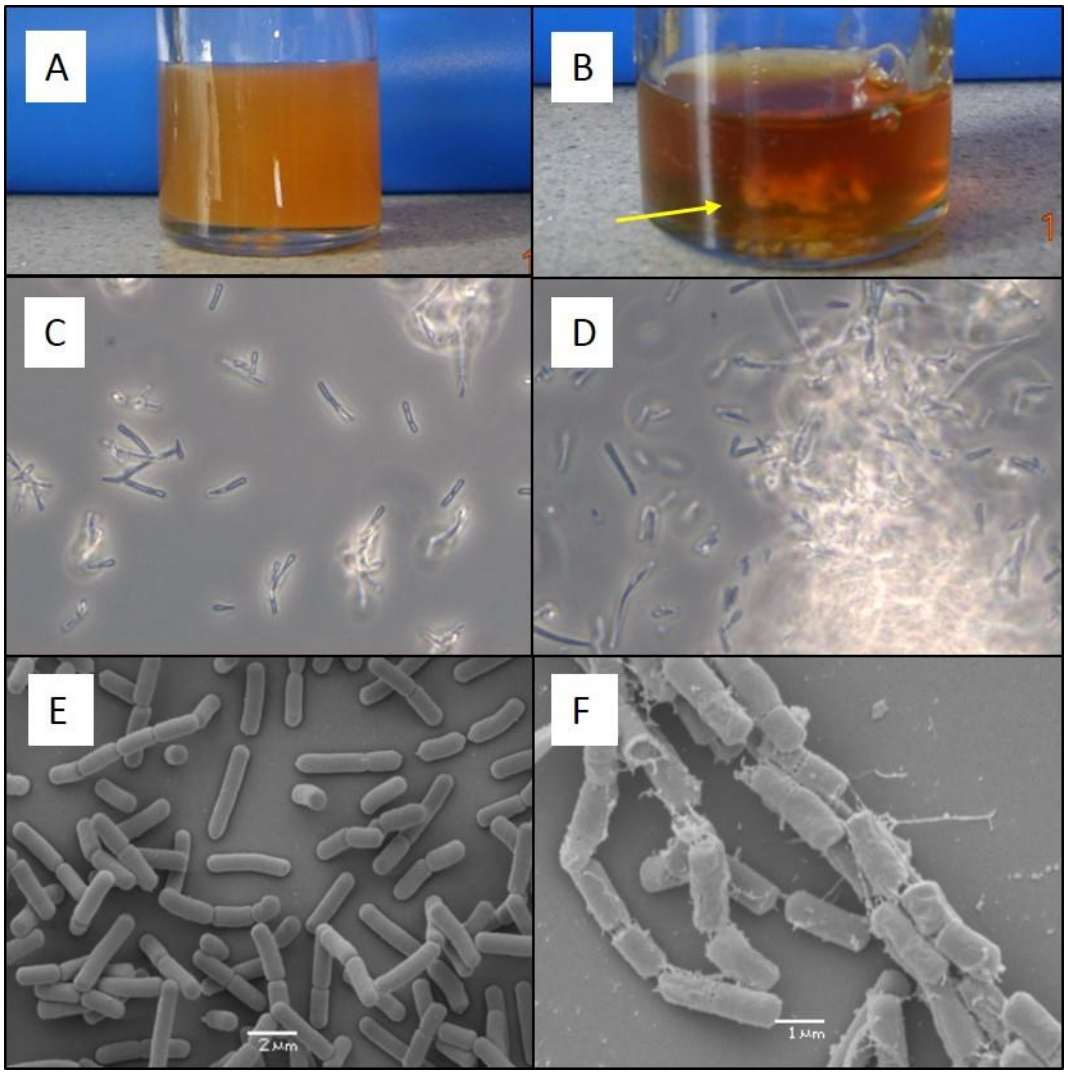


Figure 5.5 Growth of *Lactobacillus* spp. in MRS broth at 30°C and 40°C. Culture medium at 30°C (A) and 40°C (B). Cell morphology (light microscopy x 1000, Leica), of culture at 30°C (C) and 40°C (D) and when examined under the Scanning Electron Microscope 30°C (E) and 40°C (F) (Electron Microscope images were taken at Macquarie University Microscopy Unit, NSW, Australia).

5.3.5 Effect of Dunder on the Growth of *Saccharomyces cerevisiae* and *Lactobacillus* species in Molasses Medium

As mentioned previously, dunder is added to molasses for rum fermentations. Such addition could affect the growth of yeasts and bacteria associated with the fermentation. To examine this possibility, controlled fermentation experiments were conducted with molasses to which different concentrations of dunder were added. These fermentations were conducted as pure cultures with the distillers yeast, *Saccharomyces cerevisiae* and several species of *Lactobacillus* that were previously found to be associated with rum fermentations (Chapter 3). Experiments were performed in duplicate and results expressed as averages ± 6%.

The growth kinetics of the distillery yeast, *S. cerevisiae*, under varying concentrations of dunder in a molasses medium similar to that used in rum production are shown in Figure 5.6 a. In the absence of dunder, the population of *S. cerevisiae* increased from the initial inoculum level of 10^2 CFU/mL to a maximum of 1.5×10^7 CFU/mL after 40h. With the addition of 10% dunder, the growth rate of *S. cerevisiae* was decreased and the maximum population after 48 h was decreased to 4.0×10^6 CFU/mL. Yeast growth was very restricted in the presence of 25% dunder, and reached only a maximum population of 5.0×10^3 CFU/mL after 48h. In the presence of 50% dunder, no yeast growth occurred and the initial population declined to about 10^2 CFU/mL . However, the yeast cells started to recover and grow after 30 h. Doubling time and lag time, as shown in Table 5.5, increased as dunder concentration increased. The addition of 25% dunder gives the lowest specific growth rate (0.09 h^{-1}) while 10% dunder concentration gave the highest specific growth rate (during a secondary exponential phase) of 0.25 h^{-1}.

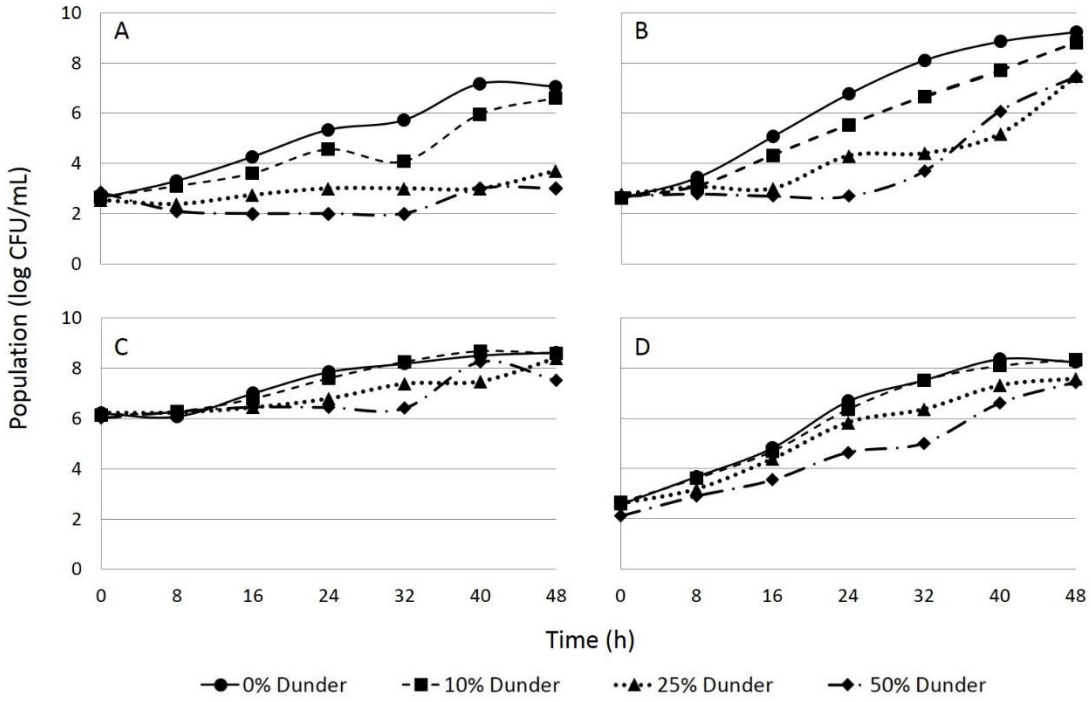


Figure 5.6 Populations of (a) *Saccharomyces cerevisiae,* (b) *Lactobacillus plantarum,* (c) *Lactobacillus fermentum* and (d) *Lactobacillus* spp. in molasses medium containing dunder at different concentrations

The impact of varying concentrations of dunder on the growth of *L. plantarum* is shown in Figure 5.6b and Table 5.5 . In the absence of dunder, *L. plantarum* populations increased exponentially to 1.7 x 10^9 CFU/mL in 48 h. The addition of dunder at levels of 10% decreased growth slightly, producing a short lag phase, and giving a final population of 6.5 x 10^8 CFU/mL at 48 h. Cultures exposed to 25% and 50% dunder experienced a significant lag phase, lasting between 16 and 24 h, respectively. After the extended lag phase, growth in the presence of 25% or 50% dunder peaked at 2.9 x 10^7 CFU/mL and 2.5 x 10^7 CFU/mL, respectively at 48 h.

Table 5.5. Effect of dunder concentration in molasses on the growth properties of *Saccharomyces cerevisiae* and several species of *Lactobacillus* .

Species	Dunder Concentration	Specific Growth Rate (h^{-1})	Doubling Time (h)	Lag Time (h)	Maximum Population (CFU/mL)
S. cerevisiae	0%	0.15[a], 0.21[b]	2.4	<8	1.15×10^7
	10%	0.13[a], 0.25[b]	2.5	<8	4.0×10^6
	25%	0.09	7	40	5.0×10^3
	50%	0.13	n/a	48	6.8×10^2
L. plantarum	0%	0.22	1.5	<8	1.7×10^9
	10%	0.15	1.5	<8	6.5×10^8
	25%	0.26	2	16	2.9×10^7
	50%	0.32	1.5	24	2.5×10^7
L. fermentum	0%	0.13	6.5	<16	4.3×10^8
	10%	0.09	4	<16	3.8×10^8
	25%	0.08[a], 0.13[b]	2	<24	2.6×10^8
	50%	0.38	1	32	3.3×10^7
Lactobacillus spp.	0%	0.25[a], 0.13[b]	2	<8	1.8×10^8
	10%	0.20	2.5	<8	2.2×10^8
	25%	0.18[a], 0.13[b]	3.1	<8	3.8×10^7
	50%	0.09[a], 0.25[b]	2	<8	2.7×10^7

[a] denotes first exponential phase

[b] denotes secondary exponential phase

Specific growth rates were calculated by linear regression of natural logarithm plot slope of cell population versus time (h) (Stanbury & Whitaker, 1984; Zwietering *et al*., 1990).

Figure 5.6c shows the impact of varying concentrations of dunder on the growth of *L. fermentum.* In the absence of dunder, *L. fermentum* populations increased exponentially to 4.3×10^8 CFU/mL by 40 h. The addition of dunder at levels of 10% affected growth marginally and final populations reached 3.8×10^8 CFU/m within 40 h. Cultures exposed to 25% and 50% dunder experienced a significant lag phase, lasting between 24 and 36 h, respectively (Table 5.5). After the extended lag phase, growth in 25% dunder gave a maximum population of 2.6×10^8 CFU/mL at 48 h. Populations of *L. fermentum* grown in 50% dunder peaked at 40 h (3.3×10^7 CFU/mL).

Figure 5.6d shows survival and growth of *Lactobacillus* spp. in varying concentrations of dunder. The growth of a *Lactobacillus* spp. in molasses medium was similar in cultures containing no dunder and 10% dunder and gave maximum populations of approximately 2.0 x 10^8 CFU/mL at 48 h. Higher concentrations of dunder did not give noticable lag phases but growth rate of the organism was decreased, more so for the 50% dunder, and final maximum populations were decreased to 2.7 x 10^7 CFU/mL.

Table 5.5 summarizes the effect of dunder on the growth rates, doubling time, lag time and maximum cell populations of *S. cerevisiae* and the different species of *Lactobacillus*.

5.3.6 Chemical Composition of Dunder

Samples of both “fresh” and “stored” dunder (see Section 5.2.1 for descriptions) were examined for pH, ethanol (%), sugars, organic acids and free amino acids (Table 5.6). Volatile compound analysis was performed on stored dunder only. Eight samples of fresh dunder and 12 samples of stored dunder collected on different occasions between 2006 and 2010 were analysed. Data presented in Table 5.6 show the ranges for these samples and averages plus the range for organic acids.

Table 5.6 Chemical properties of dunder samples taken at the Bundaberg distillery

Properties	Fresh dunder	Stored dunder	Properties	Fresh dunder mean (range)	Stored dunder mean (range)
pH	4.6 - 4.9	4.3 - 4.8	**Organic acids (mg/g)**		
Ethanol (%)	nd - 0.2	nd - 0.2	Oxalic	0.002 (0.001 – 0.002)	0.002 (0.001 – 0.002)
			Citric	0.08 (0.54 – 1.49)	1.16 (0.55 - 1.86)
Sugars (mg/g)			Tartaric	0.33 (0.09 – 0.46)	0.42 (nd – 0.73)
Sucrose	nd	nd	Malic	0.94 (0.66 – 1.29)	6.93 (1.3 – 10.3)
Glucose	nd	nd	Lactic	1.29 (0.42 – 2.01)	2.63 (1.98 – 4.42)
Fructose	nd	nd	Acetic	4.18 (0.82 – 6.25)	4.91 (2.32 – 9.85)
			Propionic	5.47 (5.36 – 5.71)	5.23 (5.09 – 5.39)
			Butyric	9.95 (9.78 – 10.11)	11.26 (11.02 – 11.88)
			Succinic	16.80 (16.57 – 18.69)	16.03 (15.89 – 16.63)

Note: nd – sugars analysis had a limit of detection of <0.1 mg/g, organic acid analysis (tartaric acid) limit of detection < 0.001 mg/g

Fresh dunder was acidic and had a pH of between 4.6 and 4.9 while stored dunder was slightly more acidic (4.3 - 4.8). There were no detectable levels of sugars (glucose, fructose or sucrose) in either stored or “fresh” dunder. The ethanol content of dunder was very low being with a maximum level of 0.02%, this being similar for both fresh and stored dunder.

5.3.6.1 Organic Acids

Nine organic acids were detected in both fresh and stored dunders. For all of the acids, there was considerable variation in the concentration of individual acid found, depending on sample. The main acids in fresh dunder were succinic followed by butyric and propionic. These were also the main acids in stored dunder

and they occurred at similar levels as found in fresh dunder. However, the stored dunder gave higher concentrations of lactic and acetic acids compared with the fresh dunder.

5.3.6.2 Amino Acids

Two samples each of fresh and stored dunder were analysed for amino acids. Results are shown in Table 5.7, with means calculated from duplicate analyses performed by the Australian Proteome Analysis Facility Ltd, Macquarie University, NSW, Australia.

Twenty one amino acids were detected in dunder, with aspartic, asparagine, alanine and glutamic having the highest concentrations (Table 5.7). For many of the acids, there were notable decreases in concentration during storage and this is reflected in a decrease in the overall total concentration of amino acids during storage. However increases were evident for alanine, methionine and ornithine.

Table 5.7 Comparison of free amino acid concentrations in fresh and stored dunder

Amino Acid (µg/mL)*	Fresh			Stored			Difference (%) [a]
	1	2	*Average*	1	2	*Average*	
Histidine	14.2	16.2	*15.2*	11.8	11.8	*12.4*	-18.4
Asparagine	93.9	652.4	*374.15*	97.7	97.7	*143.75*	-61.6
Serine	25.6	29.8	*27.7*	11.9	11.9	*17.65*	-36.3
Glutamine	9.4	4.5	*6.95*	3.4	3.4	*6.2*	-10.8
Arginine	12.0	10.5	*11.25*	5.7	5.7	*7.15*	-36.4
Glycine	408.8	21.8	*16.9*	4.4	25.7	*15.05*	-10.9
Aspartic acid	33.9	520.5	*464.65*	265.5	425.9	*345.7*	-25.6
Glutamic Acid	14.6	160.0	*96.95*	55.6	42.4	*48.9*	-49.6
Threonine	62.0	18.7	*16.65*	5.0	6.2	*5.6*	-67.0
Alanine	62.0	158.0	*110.0*	167.5	447.1	*307.3*	179.4
Proline	20.3	45.9	*33.1*	22.2	31.9	*27.05*	-18.3
Ornithine	7.3	9.7	*8.5*	3.1	23.1	*13.1*	54.1
Cysteine	1.5	1.1	*1.3*	1.0	1.2	*1.1*	-15.4
Lysine	9.1	9.6	*9.35*	4.4	4.5	*4.45*	-52.4
Tyrosine	8.6	10.7	*9.65*	8.9	nd	*4.45*	-53.9
Methionine	1.5	1.2	*1.35*	2.9	1.9	*2.4*	77.8
Valine	29.3	29.4	*29.35*	1.9	3.9	*2.9*	-90.1
Isoleucine	18.2	18.1	*18.15*	2.9	1.3	*2.1*	-88.4
Leucine	11.8	9.1	*10.45*	nd	3.0	*1.5*	-85.6
Phenylalanine	12.1	11.0	*11.55*	6.1	4.1	*5.1*	-55.8
Tryptophan	2.9	3.5	*3.2*	3.6	3.5	*3.55*	10.9
Total	809.1	1741.6	*1275.35*	685.4	1269.2	*977.3*	n/a

*Calculation based on free amino acid molecular weight.

[a] Difference % compared to fresh dunder.

Data are the averages of duplicate analyses

5.3.6.3 Volatile Compounds

As dunder is expected to contain no volatiles, due to it being the remaining liquid after boiling in the bottom of the distillation column, volatiles were only analysed in samples of stored dunder (Table 5.9). Few volatiles were found, the most significant of which were 3-methylbutanol, 1-butanol and the esters, ethyl butanoate and ethyl hexanoate.

Table 5.8 Volatile compounds identified from stored dunder

Ethyl Esters	(µg/L)
ethyl acetate	-
ethyl propanoate	-
ethyl 2-methylpropanoate	7
ethyl butanoate	137
ethyl 2-methylbutanoate	-
ethyl 3-methylbutanoate	-
ethyl hexanoate	99
Acetates	
2-methylpropyl acetate	-
2-methylbutyl acetate	37
3-methylbutyl acetate	-
2-phenylethyl acetate	15
hexyl acetate	-
Alcohols	
2-methylpropanol	-
1-butanol	2108
2-methylbutanol	-
3-methylbutanol	5857
hexanol	155

5.4 DISCUSSION

As discussed in Chapter 2, and as mentioned in several review papers (I'Anson, 1971; Fahrasmane & Ganou-Parfait, 1998; Wilkie *et al.*, 2000), dunder is a raw material that is uniquely used in rum production. The reasons for its use have probably evolved empirically over the centuries but these are briefly summarized as: recycling available fresh water, reduce distillery waste, reduce disposal costs, provide acidity required to lower molasses pH, provide a source of wild yeasts and bacteria for the next molasses fermentation. It is a by-product of each individual distillery and, consequently, its chemical and biological properties are likely to vary from one distillery to the next, depending on how it is processed and stored. There appears to be no standard procedure for its production and use from distillery to distillery. The Bundaberg distillery uses it at 7.5% volume in the fermentation medium. The reasons for its use were not clearly stated, but most likely to decrease water usage and decrease waste output, while also helping to acidify the next batch of molasses medium. The process at this distillery was managed as a pure culture yeast (*S. cerevisiae*) fermentation, and it was generally accepted that

the process proceeded to this expectation. The dunder used in the process was taken directly from the still, was assumed to be sterile and, consequently, would not give additional microbial diversity to the fermentation process. A similarly obtained by-product, “backset” stillage, has been used in whisky production (Kelsall & Piggot, 2009).

Microbiology of the dunder

Dunder samples taken directly after the still for use in preparation of the molasses fermentation medium was sterile, as expected, from the heating process. No yeasts, aerobic bacteria or anaerobic bacteria could be detected in such samples by direct plate culture (< 5 CFU/mL) or plate culture after enrichment. However under microscopic examination they showed dense quantities of yeasts cells and, to a lesser extent, bacterial cells. This is consistent with observations in previous literature (Kampen, 1975) but it is always assumed to contain dead cells.

Nevertheless, similar samples, aseptically taken and transported to UNSW were consistently fermenting on arrival (after 1-2 days by courier). Microbiological analysis of these samples, taken over an 18 month period gave isolation of the same bacterial species, identified as an “unculturable” *Lactobacillus* (GQ082129.1). While there were two different colony types, both were identified as the same species, and most likely represented different strains. This bacterial species returned the same sequencing and BLAST identification data, as well as API CHL50 profiles, that were also obtained for isolates of lactic acid bacteria described in Chapter 3 (molasses, clarifier, surge tank, yeast propagation, fermenters and buffer tanks) and Chapter 4 (buffer tanks). The isolation of this species of *Lactobacillus* from the entire production facility shows an endemic contamination and may mean that it is an important species for production efficiency or flavour development. Further research regarding the impact of this species on the fermentation of rum at the Bundaberg distillery will be described in Chapter 6.

An explanation of the development of this species in samples of dunder is puzzling. First, samples were collected aseptically which means that this species survived heating of the fermented molasses during distillation. The molasses medium is heated to 103°C at the base of the still, however this is a continuous distillation process with no specific time spent at these high temperatures. Typically, for bacterial cells to be killed, high temperatures must be maintained for particular durations, such as 15-20 min. This may not be the case during the distillation step at the Bundaberg distillery. Similarly, the high biomass density of yeast (especially) and bacterial cells at the end of fermentation, may also influence the ability of cells to survive, by conferring protection. The increased density of cells may inhibit the heat exchangers from having prolonged contact to all cells present in the fermented medium prior to distillation and may mean that some cells are left unaffected by the heating process. There is evidence that this species produces some extracellular material (Figure 5.5) that may afford some protection during the heating steps. The production of extracellular material, such as exopolysaccharides, known to occur in some lactic acid bacteria may protect the microorganism from inactivation by external factors such as attack by bacteriophages, heat or desiccation (Sutherland, 1998). The synthesis of exopolysaccharides is an important element in the production of biofilms, as the exopolysaccharides are the substances that are used to adhere the cell mass to surfaces such as plastics, soils and, in the case of the Bundaberg distillery, metal surfaces such as pipes and plates of heat exchangers (Trachoo, 2003). The ability of such microorganisms to produce biofilms would increase their ability to withstand the stresses associated with processing environments, such as heating seen at the Bundaberg distillery. Costerton *et al.* (1995), showed that biofilm forming bacteria are significantly more resistant (up to 500 times) to antimicrobial agents than non-biofilm producing cultures. Biofilms also protect microbial growth as the exopolysaccharides can form a physical barrier reducing the effectivity of biocides, such as chlorine, making CIP cleans less effective (Trachoo, 2003).

It was unusual that enrichment cultures of dunder samples taken directly after distillation did not show the presence of the *Lactobacillus* spp. although it

developed in dunder samples during transport to UNSW. Possibly, the dunder itself—rich in amino acids and vitamins, after the heating and destruction of yeasts, provides the survival and growth factors for this species. Confirmation that a heat resistant species occurs in the dunder was obtained by heating dunder and finding subsequent re-growth, although this was not consistently found. This may have been due to various factors, such as variable heat resistance of the cell or production of exopolysaccharides (as discussed previously). Further research is needed for more complete taxonomic identification and physiological characterisation of this organism but this was outside the scope and time frame of this project. The isolation of a new or novel species from this distillery environment is a most likely possibility but would not necessarily be a unique observation. Cachat & Priest (2005) isolated a novel species, *Lactobacillus suntoryeus* sp., from a malt whisky distillery and similar strains had also been isolated, in the same study, from a Japanese malt whisky fermentation.

Chemical composition of dunder

The chemical composition of dunder was described in Chapter 2, Table 2.4 where only general proximate compositions have been reported, mainly from the point of view of the chemical oxidation and biological oxidation demand properties related to waste disposal. There is little doubt that dunder is an acidic product, rich in proteins, amino acids and vitamins that would arise from heating of the high amount of yeast biomass in the fermented molasses (Basu, 1975; Bories *et al*., 1988; Wilke, 2000). The acidity of the product was confirmed in this study where the pH was between 4.3 and 4.9. This is consistent with the predominance of organic acids (Table 5.6) and is also consistent with previous reports, describing the main acids found in dunder as acetic, propionic and butyric acids (Bories *et al*., 1988). These acids would arise from the sugar processing operations that lead to the molasses, and acids produced by microbial metabolism during molasses fermentation. As shown in Table 5.7 and mentioned in earlier reports (Nicol, 2003), dunder contained significant levels of free amino acids which would serve as a source of nutrients - especially for lactic acid bacteria which are nutritionally

fastidious (Carr *et al.*, 2002). Such levels decreased during dunder storage (Table 5.7) which is consistent with the presence and growth of lactic acid bacteria in stored dunder (Table 5.2). The dunder samples examined in this study did not show the presence of any fermentable sugars, most likely as any fermentable sugars remaining after the molasses fermentation stage would probably be utilized during extended storage of the fermented product in the buffer tanks.

As dunder is expected to contain no volatiles, due to it being the remaining liquid after boiling in the bottom of the distillation column, volatiles were only analysed in samples of stored dunder (Table 5.9). Given there are hundreds of volatile compounds that have been previously characterised from rum (Appendix A), only a few key compounds were studied. However it should be noted that the presence of volatile compounds would not be limited to the few compounds highlighted in Table 5.9 and, analytically, the method chosen could have identified numerous other compounds if deemed necessary. Of those volatiles examined, few were found, the most significant of which were 3-methyl-1-butanol, 1-butanol and the esters, ethyl butanoate and ethyl hexanoate. All four of these have been previously associated with molasses based rum fermentations (Chapter 2, Table 2.6). 3-methyl-1-butanol has been found in sugar cane molasses and fermented molasses and is known to have whisky-like sensory characteristics (Lehtonen, 1983 a, b; Pino *et al* 2002). The compound, 1-butanol, previously found by Lehtonen, (1983); Pino *et al* (2002); Pino *et al*, (2012) exhibits medicinal sensory notes. Ethyl hexanoate, previously found in both Jamaican and Cuban rums, ethyl butanoate has also been found previously in Jamaican rum, and both are known to demonstrate fruity notes similar to those of pineapple (Pino *et al.,* 2002; Allan, 1972; Pino *et al.,* 2012). Work reported by Fahrasmane & Ganou-Parfait (1998) outlined the importance of *Clostridium* species in the production of higher alcohols, formic, propionic and butyric acid at the end of fermentation of molasses medium. The combination of ethanol and butyric acid (also known as butanoic acid) may lead to the production of ethyl butanoate. No *Clostridium* species were found in the ecological surveys undertaken in Chapters 3 and 4. Further research is need to determine if the

Lactobacillus spp. that develops in stored dunder is responsible for the production of these volatiles.

Effect of dunder on growth of yeasts and bacteria during molasses fermentation

As mentioned previously, one of the reasons for adding dunder to molasses rum fermentations is to serve as a potential source of nutrients for yeast growth since molasses may be deficient in assimilable nitrogen (Kampen, 1975; Fahrasmane & Ganou-Parfait, 1998). The data of Table 5.7 confirm that it is a good source of amino acids. However, the growth experiments of Figure 5.6 suggest that dunder was not necessary to promote the growth of the culture yeast *S. cerevisiae*. In contrast, dunder decreased the growth of the yeast especially at concentrations above 10%. Consequently, its use in this context could be counterproductive, and decrease fermentation efficiency - especially if other sources of nutrients, such as Fermaid, are added to the molasses medium, as is the case at the Bundaberg distillery. The use of dunder at 7.5 % at this distillery is at the borderline of having a negative impact and, therefore, its use may need re-consideration. At levels above 10%, it clearly has a negative impact on yeast growth. This negative influence could be related to the presence of relatively high levels of acetic and butyric acids which have been reported to decrease/ inhibit yeast growth (Lehtonen & Suomalainen, 1977).

The three lactic acid bacteria, isolated from the Bundaberg distillery (*L. fermentum, L. plantarum* and *Lactobacillus* spp.), also grew best when no dunder was added to the fermentation medium. Interestingly, but not surprisingly, *Lactobacillus* spp., which was isolated from dunder, grew well at all four concentrations, including 50% dunder. This is not unexpected as it can remain viable and grow in dunder samples during storage. Both *L. fermentum* and *L. plantarum* also had the potential to grow in the presence of high concentrations (25%, 50%) of dunder, but relatively long lag phases were evident compared with the *Lactobacillus* spp. (Table 5.5). This

suggests the presence of some substances in dunder that are inhibitory to their growth. Further research is necessary to determine the nature of the substances in dunder that are inhibitory to yeast and bacterial growth.

The microbiological and chemical studies of dunder have shown that it is a highly variable, acidic and complex raw material. The presumption by many researchers and distillery staff that it is sterile if collected close to the still has been put into question by the microbiological work performed in this Chapter. Its necessity as an ingredient for the supposed benefit of nutrients and growth factors is also debatable as simple growth experiments showed no added benefit for its inclusion, with potential detrimental effects to yeast growth and fermentation efficiency.

CHAPTER 6 GROWTH OF YEASTS AND BACTERIA DURING FERMENTATION OF MOLASSES FOR RUM PRODUCTION

6.1 INTRODUCTION

Although rum fermentation is performed by yeasts, primarily strains of *Saccharomyces cerevisiae*, bacteria are also present. These bacteria contribute as natural contaminants, in an uncontrolled, and largely unknown, way. The data of Chapters 3 and 4 demonstrated a consistent presence of lactic acid bacteria throughout fermentations conducted in the Bundaberg Distilling Company. Three species, *Lactobacillus fermentum, Lactobacillus plantarum,* and an unidentified *Lactobacillus spp.*, were predominant in these fermentations, frequently reaching populations as high as 10^6-10^7 CFU/mL. These populations are ecologically significant and are likely to impact on the growth and metabolic activities of *S. cerevisiae* during the fermentation and, also, have their own metabolic imprint on the production of flavour volatiles that could uniquely contribute to rum flavour and sensory character. Although such influences of lactic acid bacteria have been described for some other alcoholic fermentations such as wine (Swiegers *et al* 2005), whisky (Simpson *et al,* 2001; van Beek & Priest, 2003; Cachet & Priest, 2005; van Beek & Priest, 2000, 2001 & 2003), and cachaça (Schwan *et al,* 2001; Duarte *et al,* 2011) they have not been investigated with respect to rum production.

The objectives of this chapter are to determine the effect of these lactic acid bacteria on the growth of yeasts, process efficiency and production of flavour volatiles during rum fermentation. It will examine molasses fermentations under controlled conditions with single and mixed populations of the yeast, *S. cerevisiae*, and the lactic acid bacteria, *L. fermentum, L. plantarum* and the unidentified *Lactobacillus* spp.

6.2 MATERIALS AND METHODS

6.2.1 Species Selection

Species chosen for this investigation were isolated and identified from rum fermentations conducted at the Bundaberg Distilling Company as described in Chapter 3. These were the distillery strain of *Saccharomyces cerevisiae*, and the bacterial species *Lactobacillus plantarum*, *Lactobacillus fermentum* and the *Lactobacillus* spp. These lactic acid bacteria were also isolated and identified in Chapter 4 (from buffer tanks at the end of the fermentation process). The *Lactobacillus* spp. was also isolated and examined in detail in Chapter 5.

6.2.2 Molasses Medium and Fermentation

Molasses and dunder were obtained from the Bundaberg Distilling Company. The molasses was diluted with water to give the concentration normally used in rum fermentation, namely, 30° Brix or 15 % fermentable sugars. Part of the water was replaced with dunder to give 7.5% dunder in the final molasses medium, as used at the Bundaberg Distilling Company for rum production. The molasses medium was adjusted to pH 5.5 by addition of 5M HCl. The medium was dispensed as 8L volumes in 10L sealed stainless steel buckets with lids and sterilised by autoclaving at 121°C for 15 min.

Fermentations of the molasses medium were conducted with *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, *Lactobacillus fermentum* or the *Lactobacillus* spp. as single cultures and in combinations as shown in Table 6.1.

Table 6.1. Laboratory fermentations of molasses with inoculated yeast and lactic acid bacteria

Individual fermentations
Saccharomyces cerevisiae
Lactobacillus plantarum
Lactobacillus fermentum
Lactobacillus spp.
Mixed fermentations
Saccharomyces cerevisiae and *L. fermentum*
Saccharomyces cerevisiae and *Lactobacillus* spp.
Saccharomyces cerevisiae, L. fermentum and *Lactobacillus* spp.

Inoculum cultures of *S. cerevisiae* for the fermentations were prepared from fresh slants of MEA and cultured in MEB at 30°C for 24-48 h, while inoculum cultures of individual bacteria were prepared from slants of MRS agar and cultured into MRS broth at 30°C for 24 h. Each of these cultures (5 mL) were inoculated into separate 100 mL of molasses medium and incubated at 30°C for 24-36 h. This culture was used to inoculate individual 500 mL of molasses medium and incubated for a further 24 h at 30°C. The 500 mL volume of culture was then used to inoculate the 8 L of molasses medium for the fermentation trials.

The fermentations were incubated at 30°C for 48 h without shaking and samples (100 mL) were aseptically taken every 8 h until 48 h. These samples were used immediately for microbiological analyses as described in Section 6.2.4 and the remaining part of the sample was stored at -20°C until chemical analysis. Each of the fermentation trials listed in Table 6.1 was conducted in duplicate.

6.2.3 Distillation of Fermented Molasses

Distillation at the Bundaberg distillery, was described in Chapter 3, Section 3.3.1.6. To attempt to replicate the two stage process undertaken, laboratory distillation was first done in a custom made distillation column. This was known as the “primary” distillation. The distillate collected from this primary distillation was then subjected to further distillation using laboratory distillation glassware to replicate the “secondary” pot still distillation undertaken at the Bundaberg distillery.

6.2.3.1 Primary Distillation

Distillation was undertaken, using a custom made distillation column, designed to simulate critical properties of the primary distillation process at the Bundaberg Distilling Company. This distillation column (Figure 6.1) was constructed by the Workshop, School of Chemical Engineering, UNSW.

The distillation unit consisted of a stainless steel vessel (Fig 6.1 a) that had an electric heating element (Fig 6.1 b) inserted directly through the side of the vessel. The height at which this heating element was located, restricted the vessel to a minimum operational volume of 6L. The distillation column (Fig 6.1 d) was mounted on top of this vessel. Components of the distillation unit were joined using stainless steel connection flanges (Fig 6.1 i) with a heat stable rubber seal used between each component to ensure the column was airtight.

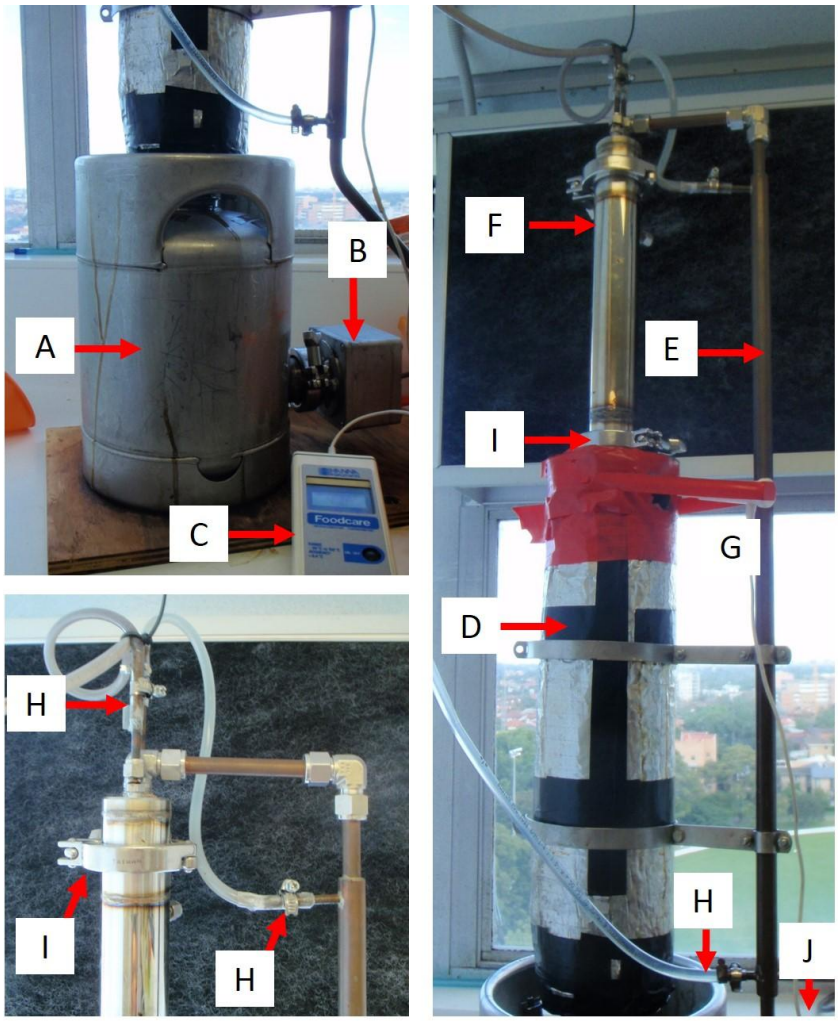


Figure 6.1 Components of the batch distillation column used for large scale distillation of fermented molasses. (a) stainless steel vessel, (b) electric heating element unit, (c) thermocouple readout display, (d) stainless steel column with fibre glass insulation, (e) external copper condenser (water cooled), (f) stainless steel reflux section with internal copper coiled condenser (water cooled), (g) thermocouple port, (h) inlet and outlet of cooling water supply, (i) stainless steel connection flanges

The stainless steel column had a 1/8” Swagelok fitting halfway along its length, which acted as a thermocouple port allowing the temperature of the column to be monitored (Fig 6.1 c, g). The column had 31 mm thick fibreglass insulation around its circumference (Fig 6.1 d), which prevented the rising vapour from condensing on the inside walls and allowed the column to reach thermal equilibration efficiently.

An extension of the main section of the column was mounted on the top with an internal copper coiled condenser (Fig 6.1 f). The presence of the internal condenser meant that the extended section of the column was under reflux.

The outlet at the top of the reflux section was attached to a second external copper condenser (Fig 6.1 e) which acted as an added quality and safety precaution, preventing the loss of any vapour from the top of the column. Both condensers were water cooled, which was carried under pressure from the laboratory tap using 10 mm PVC piping (Fig 6.1 h). Since the flow rate through the internal condenser needed to be controlled, the in-flow of water was fed through a rotameter (not shown), which allowed the rate to be easily manipulated and held constant.

The distillate released from the external condenser was collected in Schott bottles which were sitting in ice (Fig 6.1 j). Distillates were collected and stored in air tight containers prior to undergoing secondary distillation (Section 6.2.3.3) and then analysis of volatile compounds.

Fermented molasses medium (48 hr sample, 8 litres) was transferred to the base vessel. Fermentation solids, including spent yeasts, were not separated and were transferred into the vessel. Distillation was commenced by turning on the heating element, where the temperature increased to approximately 100°C The ethanol concentration of the distillate could be estimated from the thermocouple reading at the top of the internal condenser (Fig 6.1 g) using standard chemical data from the equilibration between the liquid and vapour phases of an ethanol solution at varying temperatures (CRC, 1977). In these calculations, the temperature of condensation was simply taken as the thermocouple reading at the top of the internal condenser. The final temperature required at the top of the internal condenser to give an ethanol concentration of about 50% (v/v) was 93.5- 94°C. As such, distillation was considered to be complete once the temperature reached 93.5°C. Time was not used as a method of measuring completion as time would vary depending on the composition of the ferment. The volume of distillate varied depending on the microbiological content of the ferment, *S. cerevisiae* containing ferments returned volumes of between one and two litres while bacteriological

based ferments only produced 200-500mL of distillate. The distillates were stored in Schott bottles, wrapped with parafilm to ensure an airtight seal.

6.2.3.2 Secondary Distillation

Distillates obtained from the primary distillation (6.2.3.2) were subject to a secondary distillation step to refine the distillate. This done was to simulate the two step process of distillation at the Bundaberg distillery. Distillation was performed using scientific glassware for laboratory distillations, representing a pot distillation. A 1 L round bottom reaction flask (Fig 6.2 a) was used as the pot, heated with a heating mantle (Fig 6.2 b). A thermometer was attached to monitor the temperature of evaporated primary distillate (Fig 6.2 c). The condenser (Fig 6.2 d), with continuous flowing cold water (Fig 6.2 e), was attached and condensate was collected at specific cuts, based on temperatures, in a receiver flask sitting in ice (Fig 6.2 f).

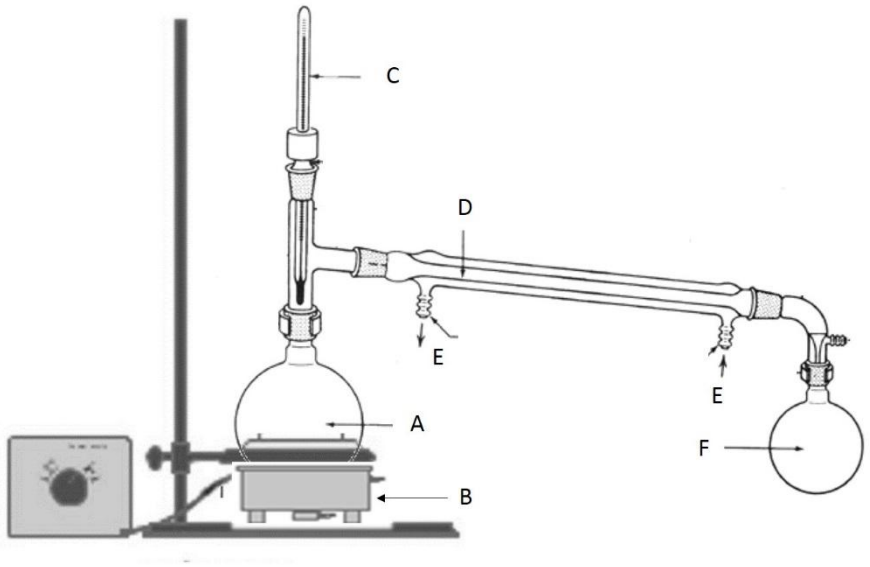


Figure 6.2 Secondary distillation apparatus used for distillation of primary distillate. (a) reaction flask, (b) heating mantle, (c) thermometer, (d) condenser, (e) cooling water, (f) receiver flask

Samples obtained from the primary distillation stage underwent further separation. Separation was, as previously mentioned, monitored via temperature. Distillates collected prior to 78°C were collected as heads, those collected between 78°C and 89°C were the “hearts” and above 89°C represented the “tails”. Distillates were stored in glass vessels. The hearts were analysed for volatile compounds (method described in further detail in Section 6.2.5.4).

6.2.4 Microbiological Analyses

Yeast growth was monitored by spread inoculating 0.1 mL of serially diluted samples, in duplicate, onto plates of MEA (Oxoid) (supplemented with 100 µg/mL oxytetracycline for mixed fermentations). Bacterial growth was monitored by spread plating 0.1 mL of serially diluted samples, in duplicate, onto plates of MRS agar (Oxoid) (supplemented with 10 µg/mL cycloheximide for mixed fermentations). Inoculated plates were incubated for 48 hours at 30°C after which colonies were counted. In mixed culture fermentations, the colonies of the different bacterial species were readily distinguishable by their morphology on MRS agar as reported in Chapter 3.

6.2.5 Chemical and Physical Analyses

6.2.5.1 Sample Preparation

Samples of fermented molasses medium for chemical analyses were clarified by centrifugation at 4°C 20,000g for 20 min (Beckman-Coulter, Fullerton, CA) and the supernatant collected. The supernatant was then filtered under vacuum through a 0.45 µm filter membrane (Millipore). Samples were stored, at -20°C until analysed. The filtration step was not done for samples used for the analysis of ethanol and volatile compounds.

6.2.5.2 pH and Brix

These analyses were done on unclarified samples taken directly from the fermentations using an Activon© optical pH probe for pH and a PAL-1 (ATAGO,

Japan) refractometer for Brix. Analyses were done in duplicate and average values are reported.

6.2.5.3 Sugars, organic acids and ethanol

The concentrations of sugars and organic acids were determined by HPLC as described in Chapter 5, Section 5.2.6. A coefficient of variation for both the sugars and organic acid methods was a maximum of ± 25% for each analysis.

Ethanol concentrations were determined using an enzymatic UV assay kit with appropriate standards and control as described by the manufacturer (Cat no. 10176290035; r-biopharm, Roche, Germany). Analyses were done in duplicate and average values are reported.

6.2.5.4 Volatile Compounds

Two methods of analysis for volatile compounds were performed. The first method was a detailed method, able to analyse both fermented molasses samples and distillates. The second method analysed only distillates and was performed on equipment used by laboratory staff at the Bundaberg distillery.

Method 1

Volatile compounds were measured in samples of fermented molasses taken directly from fermentation vessels and distillates of fermented samples.

Volatiles in samples were measured by head space analysis at the commercial facility of the Metabolomics Unit of the Australian Wine Research Institute (AWRI), South Australia. Samples were optimized for analysis by dilution in 10% potassium hydrogen tartrate buffer, pH 4.5.

The analysis was performed on an Agilent 7890 gas chromatograph equipped with Gerstel MPS2 multi-purpose sampler and coupled to an Agilent 5975C VL mass selective detector. Instrument control and data analysis were performed with Agilent G1701A Revision E.02.00 ChemStation software. The gas chromatograph was fitted with a 30 m x 0.18 mm Resteck Stabilwax – DA (crossbond carbowax

polyethylene glycol) column with a 5m x 0.18 mm retention gap. Helium (Ultra High Purity) was used as the carrier gas with linear velocity 24.6 cm/s, flow-rate 0.78 mL/min in constant flow mode. The oven temperature started at 33°C, was held at this temperature for 4 min, then increased to 60°C at 4°C/min, followed by heating to give increases of 8°C/min to 230°C when it was held for 5 min.

The conditions of large volume headspace sampling were as follows: The vial and its contents were heated to 40°C for 10 minutes with agitation (speed 750 rpm, on time 80 s, off time 1 s). A heated (55°C) 2.5 mL syringe penetrated the septum (27.0 mm) and removed 2.5 mL of headspace (fill speed 200 µL/s). The contents of the syringe were then injected into a Gerstel PVT (CIS 4) inlet fitted with a Tenax TA inlet liner (0.75 mm I.D., pre-conditioned in the GC inlet at 200°C for 1 hour and then ramped to 350°C to remove contaminates prior to first injection). Prior to injection, the inlet was cooled to 0°C with liquid nitrogen. The sample was introduced at 25.0 µL/s (penetration 22.0 mm) using split mode (split ratio 33:1, split flow 25.78 mL/min). Following capture of analytes on the Tenax liner, the injector was heated to 330°C at 12°C/min (pressure 24.6 kPa).

The mass spectrometer conditions were as follows: the quadrupole temperature was set at 150°C, the source was set at 250°C and the transfer line was held at 280°C. Positive ion electron impact spectra at 70eV were recorded in selective ion monitoring (SIM) mode and Scan mode simultaneously (relative EM volts) with a solvent delay of 4.0 min. Quantitative data processing was performed with Agilent G1701A Revision E.02.00 ChemStation software. Samples were prepared and analysed in a randomized order with a blank run every 10 samples. Analyses were done in duplicate and average values are reported.

Method 2

Methyl salicylate concentrations were measured in samples of distillates obtained during distillation in the laboratory, with a sample of commercially available matured Bundaberg Rum run as a comparative sample.

Samples were analysed using a Varian ® CP-3800 Gas Chromatograph, coupled with a Flame Ionisation Detection system (GC-FID). A Varian ® fused silica, CP-WAX 52CB column (30 m x 0.53 mm) was used with nitrogen as the carrier gas. For each analysis, 1.0µL of sample was injected into the column at 250°C (split ratio 30). The temperature program was as follows; initial 30°C for 9 min, ramp 20°C/min to 70°C and hold for 3 min, ramp 25°C/min to 190°C and hold for 5 min. A final ram of 20°C/min to 220 and a final hold for 2.5 min. Simultaneously, pressure was also adjusted automatically using the following program; initial pressure 3psi for 9 min, increase 2 psi/min to 5 psi, hold for 14 min and a final increase by 2 psi/min to 9 psi and hold for 1.8 min. The FID operated at 300°C.

6.3 RESULTS

6.3.1 Growth of Individual Cultures of *S. cerevisiae* and Species of Lactic Acid Bacteria

Saccharomyces cerevisiae and the species of lactic acid bacteria were inoculated into the molasses medium at initial levels of approximately 10^6 CFU/mL to simulate what is likely to occur in the commercial operations at the Bundaberg distillery, and their growth profiles followed during 48 h of fermentation.

Figure 6.3 shows the growth of single cultures of the different species in molasses medium. Data presented are the averages of analyses from the duplicate fermentations. The raw data for these fermentations are given in Appendix B. *Saccharomyces cerevisiae* grew from initial populations of about 6 x 10^5 CFU/mL to maximum populations of approximately 10^7 CFU/mL at the end of fermentation. The main fermentable sugar of the medium was sucrose at initial concentrations of 127 mg/g. It was progressively utilized during fermentation, in parallel with the growth of the yeast and production of ethanol. It was not completely utilized and residual levels of about 54 mg/ml were present at the end of fermentation (Fig 6.3 a). Smaller amounts of glucose (62 mg/g) and fructose (54 mg/g) were present in the medium and were partially utilized throughout fermentation. As sugars were

utilised, the ethanol content increased from about 0.0% to 5.1% w/v. The initial amount of ethanol in the medium came from the starter inoculum. The fermentation medium became marginally more acidic, with pH changing from 5.3 to a final pH of 5.2.

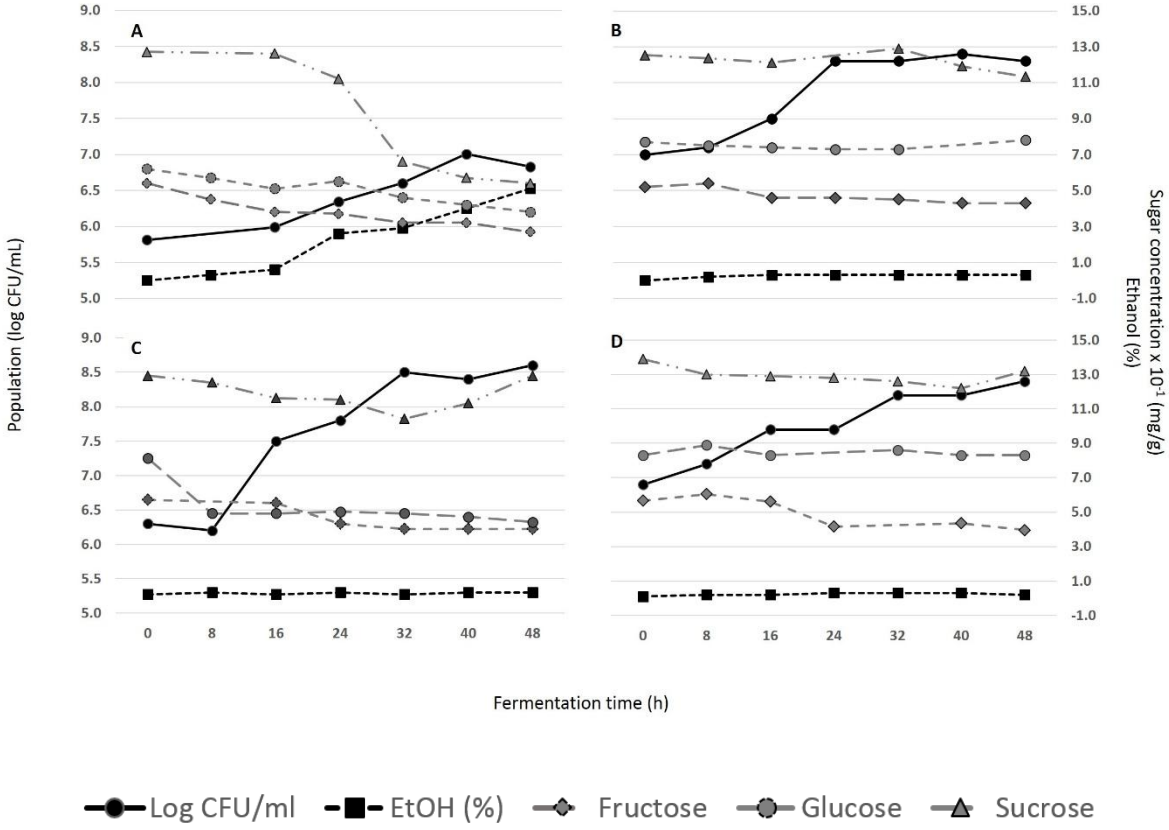


Figure 6.3 Growth profiles and changes in concentrations of fructose, glucose, sucrose and ethanol. during fermentation of molasses medium with (a) *S. cerevisiae*, (b) *L. fermentum* (c) *L. plantarum* (d) *Lactobacillus* spp. Data are the mean values of analyses from duplicate fermentations. Standard errors were less than ±6% (population log CFU/mL), ±15% (ethanol) and ±25% (sugars) of the mean values.

Lactobacillus fermentum (Figure 6.3 b), *L. plantarum* (Figure 6.3 c) and the *Lactobacillus* spp. (Figure 6.3 d) grew from initial populations of about 10^6-10^7 CFU/mL to maximum levels of approximately 5 x 10^8 CFU/mL. Despite this growth, the fermentable sugars, sucrose, glucose and fructose were only slightly utilised during fermentation (initial total sugar concentrations of about 260 mg/g to a final total sugar concentration of about 220 mg/g). In addition, the fermentations for *L.*

plantarum produced some unusual data such as a small increase in fructose levels after 8 hr of fermentation, followed by its utilisation, and an increase in sucrose concentration towards the end of fermentation. These same trends were found in each of the duplicate fermentations (Appendix B). The concentration of ethanol increased from an initial value of approximately 0.0% to only 0.3% by 48 h. During these bacterial fermentations, the pH became more acidic, decreasing from about pH 5.3 to 4.2 by 48 h for all three species.

6.3.2 Growth of Mixed Cultures of *S. cerevisiae* and Species of Lactic Acid Bacteria

Molasses medium was fermented with mixed cultures of *S. cerevisiae* and different species of lactic acid bacteria as given in Table 6.1. Figure 6.4 shows the growth of these mixed cultures of the different species in molasses medium. Data presented are the averages of analyses from the duplicate fermentations. The raw data for these fermentations are given in Appendix B.

Figure 6.4 (a) shows the mixed culture of *S. cerevisiae* and *L. fermentum.* The populations of *S. cerevisiae* increased from initial values of about 10^6 CFU/mL at the beginning of fermentation to about 10^7 CFU/mL at the end of fermentation and those of *L. fermentum* increased from approximately 10^7 CFU/mL to greater than 10^8 CFU/mL. This growth was accompanied by an almost complete utilization of both fructose and glucose and partial utilization of sucrose. As sugars were utilised, the ethanol content increased from 0.1 % to 7.9 %. The fermentation medium became slightly more acidic, with pH changing from 5.2 to 4.7.

A mixed culture of *S. cerevisiae* and the *Lactobacillus* spp. is shown in Figure 6.4(b). Populations of the yeast increased from about 10^6 CFU/mL to about 10^7 CFU/mL and those the *Lactobacillus* spp. increased from approximately 10^7 CFU/mL to a final population of 5.0×10^8 CFU/mL. Glucose and fructose were only partially utilized (fructose 51 mg/g – 26 mg/g; glucose 60 mg/g – 31 mg/g) during fermentation. Sucrose levels decreased progressively from initial values of approximately 140 mg/ml to 80 mg/ml. Ethanol content rose, in conjunction with the

sugar utilisation, from 0.1% to final content of 6.0%. The fermentation medium became slightly more acidic, with pH changing from 5.2 to 4.5.

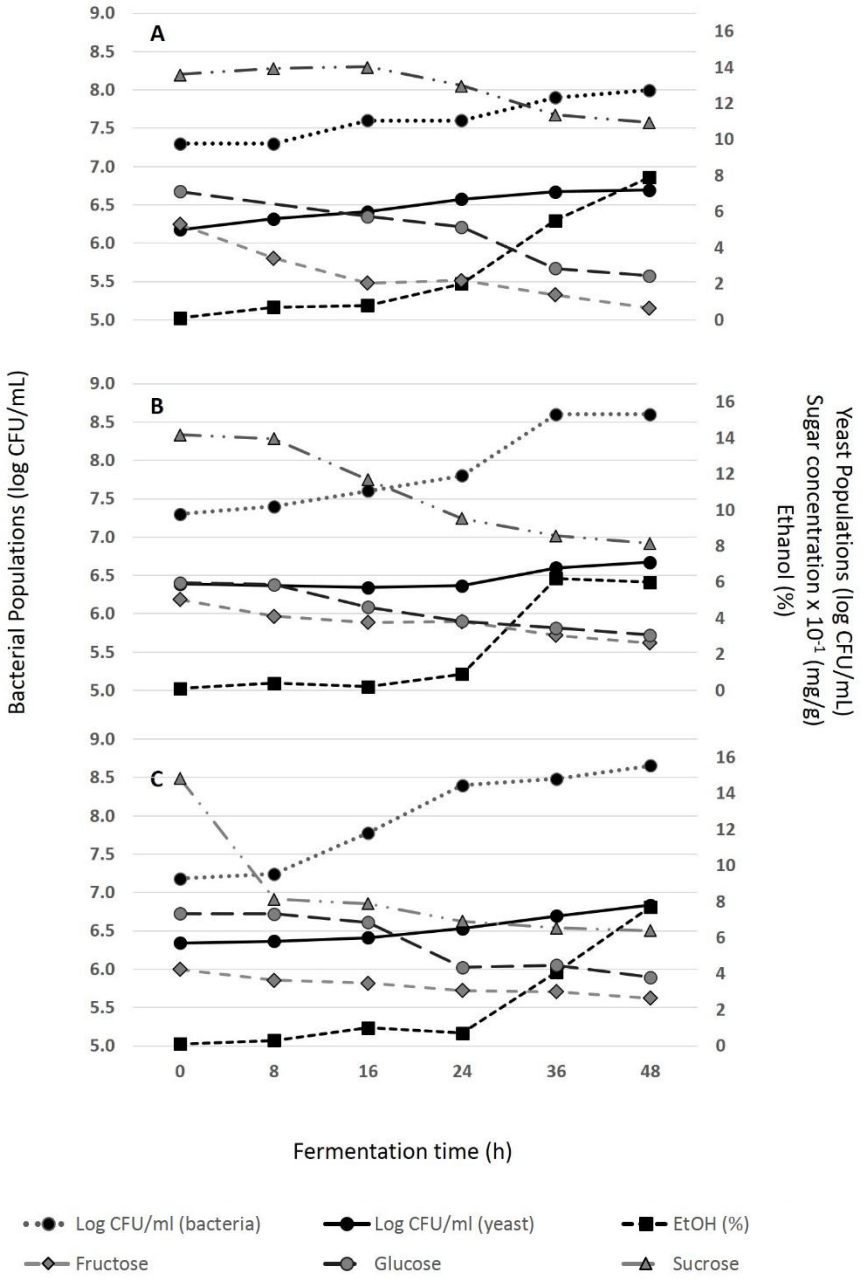


Figure 6.4 Growth profiles of mixed culture fermentations of molasses medium (a) *S. cerevisiae* and *L. fermentum*, (b) *S. cerevisiae* and *Lactobacillus* spp. (c) *S. cerevisiae, L. fermentum* and *Lactobacillus* spp. showing changes in concentrations of fructose, glucose, sucrose and ethanol.

The mixed culture, consisting of *S. cerevisiae* and both lactic acid bacteria (*L. fermentum* and *Lactobacillus* spp.) is shown in Figure 6.4 (c). Populations of *S. cerevisiae* increased from about 10^6 CFU/mL to 6.3 x 10^7 CFU/mL after 48 h. Total bacterial populations were 1.6 x 10^7 CFU/mL initially and increased to above 5 x 10^8 CFU/mL at the completion of fermentation. Individual bacterial populations were not determined. Sucrose was progressively utilised throughout the fermentation from initial concentrations of 148 mg/g to final concentrations of 64 mg/g. Fructose was partially utilised (initial concentration 43 mg/g) with a final concentration of 27 mg/g. Glucose concentrations also decreased, in conjunction with the other sugars, from 73 mg/g to a final concentration of 38 mg/g. Ethanol content increased from 0.1% to a final level of 7.7% after 48 h. As with all previous fermentations, the fermentation medium became slightly acidic, with pH changing from 5.2 to 4.2.

6.3.2.1 Organic acids

Samples taken during the laboratory scale fermentations performed above (Section 6.3.1 & 6.3.2) were analysed for organic acids. At the beginning of fermentation, the fermentation medium contained oxalic acid at 0.28 ± 0.08 mg/mL; citric acid at 1.60 ± 0.8 mg/mL; tartaric acid at 3.33 ± 1.2 mg/mL; malic acid at 4.28 ± 3.8 mg/mL; lactic acid 3.27 ± 0.7 mg/mL; acetic acid 2.01 ± 0.36 mg/mL; propionic acid at 0.23 ± 0.18; butyric acid 9.46 ± 2.5 mg/mL; and succinic acid 20.81 ± 2.0 mg/mL. There were considerable variations in determining the concentrations of these organic acids despite repeated assays. Possibly, some constituents in the molasses were causing interference with the HPLC method used. One-way single factor analysis of variance and *t*-test were used to determine significant differences between means using Microsoft Excel. Significant differences in the concentrations of organic acids were considered when $p<0.05$. Appendix C shows the raw data with notations on all results that had significant changes ($p<0.05$)

Figure 6.5 shows changes in the concentrations of organic acids during fermentations with individual cultures of *S. cerevisiae* and the species of lactic acid

bacteria. The concentrations of oxalic, citric, tartaric and propionic acids did not show major changes during fermentation and are not shown in the figure.

Fermentations undertaken by, *S. cerevisiae*, showed a decrease in malic acid at 48 hours while lactic, acetic, butyric and succinic acids all showed small but fluctuating increases between 24-48 h. The *L. fermentum* fermentation gave decreases in malic and succinic acids but only the 24 h data were statistically significant. There were increases in concentrations of lactic and butyric acids at late stage fermentation (≥24 h). Both *L. plantarum* and *Lactobacillus* spp. fermentations gave decreases in succinic acid concentrations and increases in the concentrations of lactic and acetic acids. *Lactobacillus* spp. fermentation, unlike the other three fermentations, had a significant decrease of butyric acid (48 h).

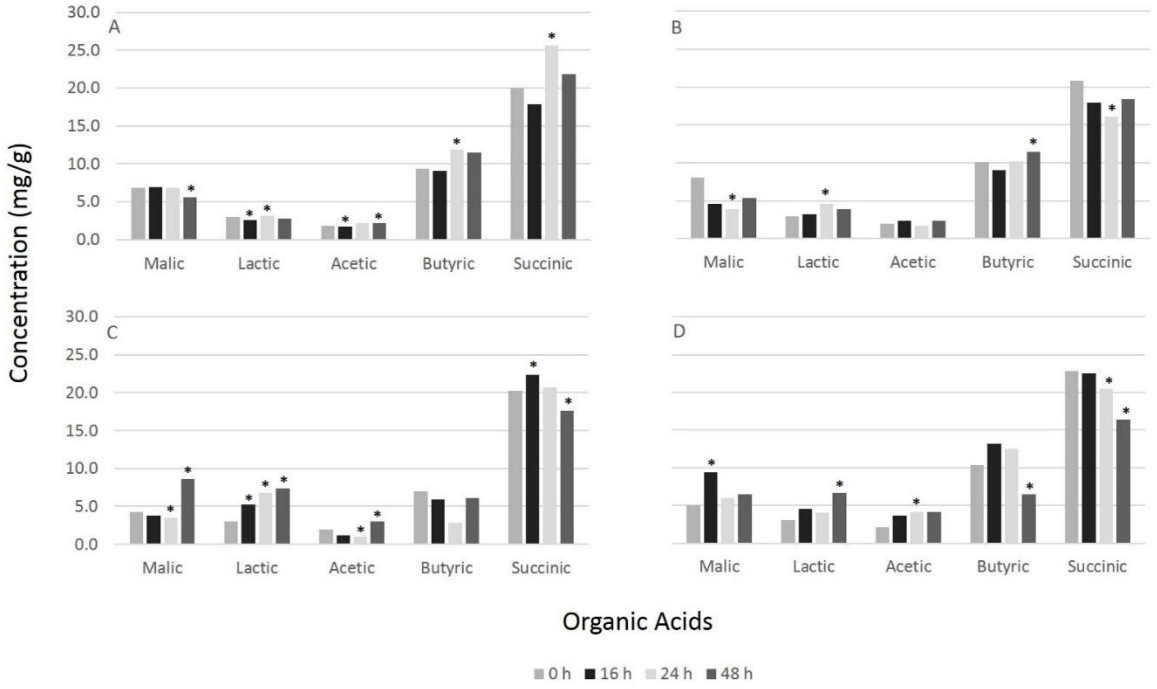


Figure 6.5 Fermentation of molasses medium by individual microbial species (a) *S. cerevisiae*, (b) *L. fermentum* (c) *L. plantarum* (d) *Lactobacillus* spp. showing changes in concentrations of five organic acids (malic, lactic, acetic, butyric and succinic) at 4 time stations (0h, 16h, 24h, 48h). One-way single factor analysis of variance and *t*-test were conducted and (*) indicates significant differences between T = 0h and subsequent time points, at a 95% confidence level.

Changes in the concentrations of organic acids during mixed culture fermentations are shown in Figure 6.6. There were clear increases of lactic, butyric and succinic acids in all three fermentations, although not always statistically significant.

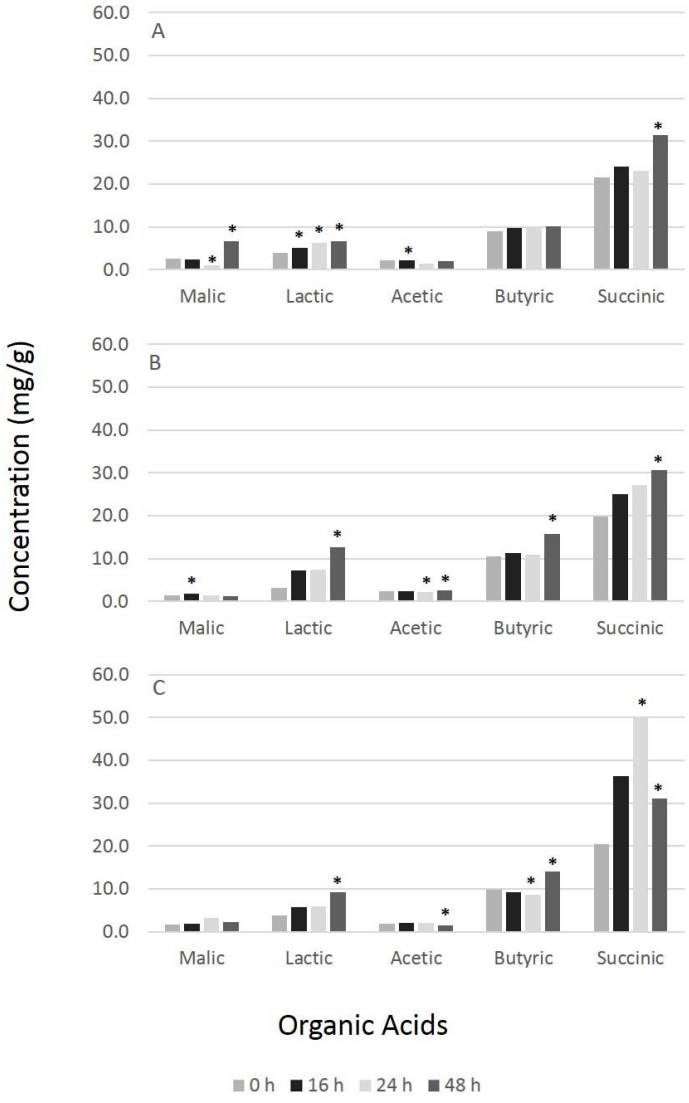


Figure 6.6 Fermentation of molasses medium by mixed microbial cultures (a) *S. cerevisiae* & *L. fermentum,* (b) *S. cerevisiae* & *Lactobacillus* spp. (c) *S. cerevisiae, L. fermentum* & *Lactobacillus* spp. showing changes in concentrations of five organic acids (malic, lactic, acetic, butyric and succinic) at 4 time stations (0h, 16h, 24h, 48h). One-way single factor analysis of variance and *t*-test were conducted and (*****) indicates significant differences between T = 0h and subsequent time points, at a 95% confidence level.

6.3.2.2 Volatile compounds

The concentrations of volatile compounds produced during the laboratory fermentations were determined in (a) samples of the fermentation culture taken at the mid-stage (16h/24h) and end (48h) of fermentation and (b) in distillates of the fermented medium at 48 h.

Table 6.2 shows some main volatiles produced during molasses medium fermentation by single and mixed cultures *S. cerevisiae* and lactic acid bacteria.

The main volatiles (apart from ethanol, discussed previously) produced during fermentation were the higher alcohols; 3-methylbutanol, 2-methylpropanol and 2-methybutanol. These were principally produced by *S. cerevisiae* (Figure 6.7) with very little produced by either of the three species of lactic acid bacteria, especially when the basal levels of these alcohols in the fermentation medium (0 h data) are taken into consideration. These three higher alcohols were also predominant in the mixed yeast- lactic acid bacteria fermentations, although the mixed fermentations with *Lactobacillus* spp. and both *L fermentum* and *Lactobacillus* spp. decreased the levels of 2-methylbutanol and 3-methylbutanol by about 50%.

Ethyl acetate and ethyl propanoate were the main esters found in these ferments, being produced almost exclusively by *S. cerevisiae* (Table 6.2). Single cultures of the lactic acid bacteria did not produce these acetates or other esters. 3-methyl butyl acetate and phenyl ethyl acetate were also measured but were not detected in any of the fermentations except trace amounts for the late stage of the *S. cerevisiae* fermentation or if combined with the bacteria in fermentation. Production of ethyl acetate and ethyl propanoate also occurred in the mixed culture fermentations with some apparent increases for ethyl acetate in these cases. Insufficient samples were analysed to make firm conclusions about these increases.

Table 6.2 Some volatiles produced during molasses medium fermentation by single and mixed cultures of *S. cerevisiae* and lactic acid bacteria

Sample		Alcohols (µg/L)					Ethyl Esters (µg/L)			
Starter culture	Time station (h)	2-methyl propanol	1-butanol	2-methyl butanol	3-methyl butanol	hexanol	Ethyl acetate	Ethyl propanoate	Ethyl butanoate	Ethyl hexanoate
Fermentation medium, no culture		648	-	-	5824	49		-		-
S. cerevisiae	16	2001	-	797	7491	49	-	5	-	-
	48	11996	815	9809	29462	88	479	69	8	-
L. fermentum	16	975	30	44	5981	102	-	-	-	-
	48	1746	526	256	6299	123	-	-	-	-
L. plantarum	16	782	-	-	5764	51	-	-	-	-
	48	1069	-	148	6271	77	7	-	-	-
***Lactobacillus* spp.**	16	1093	78	213	6140	131	-	-	-	-
	48	2003	607	368	6490	130	-	-	-	-
S. cerevisiae* & *L. fermentum	24	3766	38	2374	11850	48	60	-	-	-
	48	14162	790	10151	33972	88	767	11	11	-
S. cerevisiae* & *Lactobacillus spp	24	1651	-	647	7121	54	-	-	-	-
	48	7692	790	5897	21618	142	578	-	9	35
S. cerevisiae*, *L. fermentum* & *Lactobacillus spp	24	3290	68	1637	9144	101	-	-	-	-
	48	7219	814	4768	19335	169	640	27	7	36

-below limit of quantification

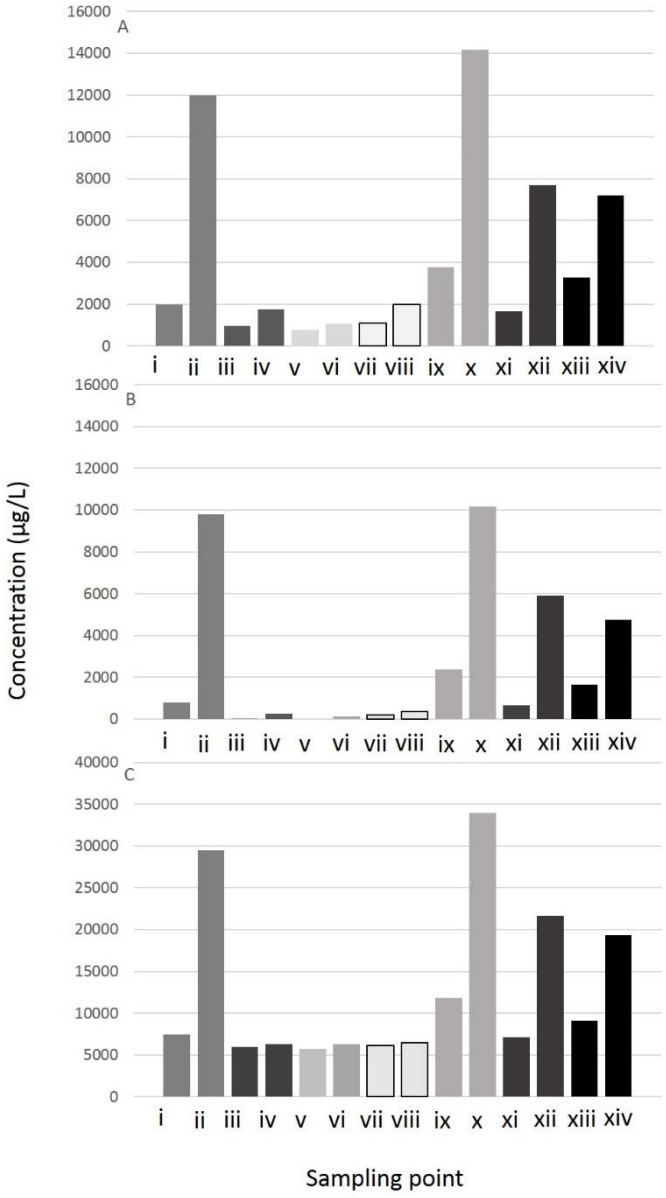


Figure 6.7 Key alcohols (A) 2-methylpropanol, (B) 2-methylbutanol and (C) 3-methylbutanol, produced during molasses medium fermentation by single and mixed cultures. (i) *S. cerevisiae* (16 h), (ii) *S. cerevisiae* (48 h), (iii) *L. plantarum* (16 h), (iv) *L. plantarum* (48 h), (v) *L. fermentum* (16 h), (vi) *L. fermentum* (48 h), (vii) *Lactobacillus* spp. (16 h), (viii) *Lactobacillus* spp. (48 h), (ix) *S. cerevisiae* & *L. fermentum* (24 h), (x) *S. cerevisiae* & *L. fermentum* (48 h), (xi) *S. cerevisiae* & *Lactobacillus* spp. (24 h), (xii) *S. cerevisiae* & *Lactobacillus* spp. (48 h), (xiii) *S. cerevisiae, L. fermentum* & *Lactobacillus* spp. (24 h), (xiv) *S. cerevisiae, L. fermentum* & *Lactobacillus* spp. (48 h).

As mentioned in Section 6.2.5.4, distillates of the laboratory fermentations were analysed for volatiles by two methods, one as used at the AWRI (to determine levels of specific alcohols, ethyl esters and acetates, known to be important in rum

flavour and composition). and the other as routinely used by the quality assurance laboratory at the Bundaberg distillery to detect, among other volatile compounds, the specific presence of methyl salicylate, a methyl ester that the Bundaberg distillery deemed to be of importance in the flavour of Bundaberg Rum.

Table 6.3 Higher alcohols detected in the distillates of laboratory fermentations of molasses medium with combinations of *Saccharomyces cerevisiae* and species of lactic acid bacteria

Sample	Alcohols (µg/L)				
Starter culture	2-methyl propanol	1-butanol	2-methyl butanol	3-methyl butanol	hexanol
S. cerevisiae	113024	7105	90196	220906	422
L. fermentum	31599	15372	14548	25769	2270
L. plantarum	300291	6754	209481	566322	7008
***Lactobacillus* spp.**	28162	12706	12432	22453	1747
S. cerevisiae* & *L. fermentum	135117	7718	102468	280548$	692
***S. cerevisiae* & *Lactobacillus* spp**	56371	6106	46204	124944	783
***S. cerevisiae*, *L. fermentum* & *Lactobacillus* spp**	42058	4378	26621	79127	507
Bundaberg Rum	91552	9382	93009	364888$	900

-below limit of quantification
$ above calibration limit

The main higher alcohols found in the distillates were 2-methylpropanol, 2-methylbutanol and 3-methylbutanol, with lesser amounts of butanol and hexanol (Table 6.3). With the exception of butanol, the highest amounts of these alcohols were found in distillates of the fermentation with *L. plantarum*, followed by *S. cerevisiae* as illustrated in Figure 6.8. The lowest amounts were present in distillates of fermentations with *L. fermentum* and *Lactobacillus* spp. Distillates from the combined fermentations of *S. cerevisiae* with *Lactobacillus* spp. or *S. cerevisiae, L. fermentum* and *Lactobacillus* spp. gave lesser amounts of these alcohols than that from a single *S. cerevisiae* fermentation, but higher amounts

were found in distillates of from the *S. cerevisiae* and *L. fermentum* fermentation. Parallel analyses of samples of a finished product of Bundaberg rum confirmed the predominance of 3-methyl butanol, 2-methyl butanol and 2-methyl propanol in this product along with lesser amounts of 1-butanol and hexanol.

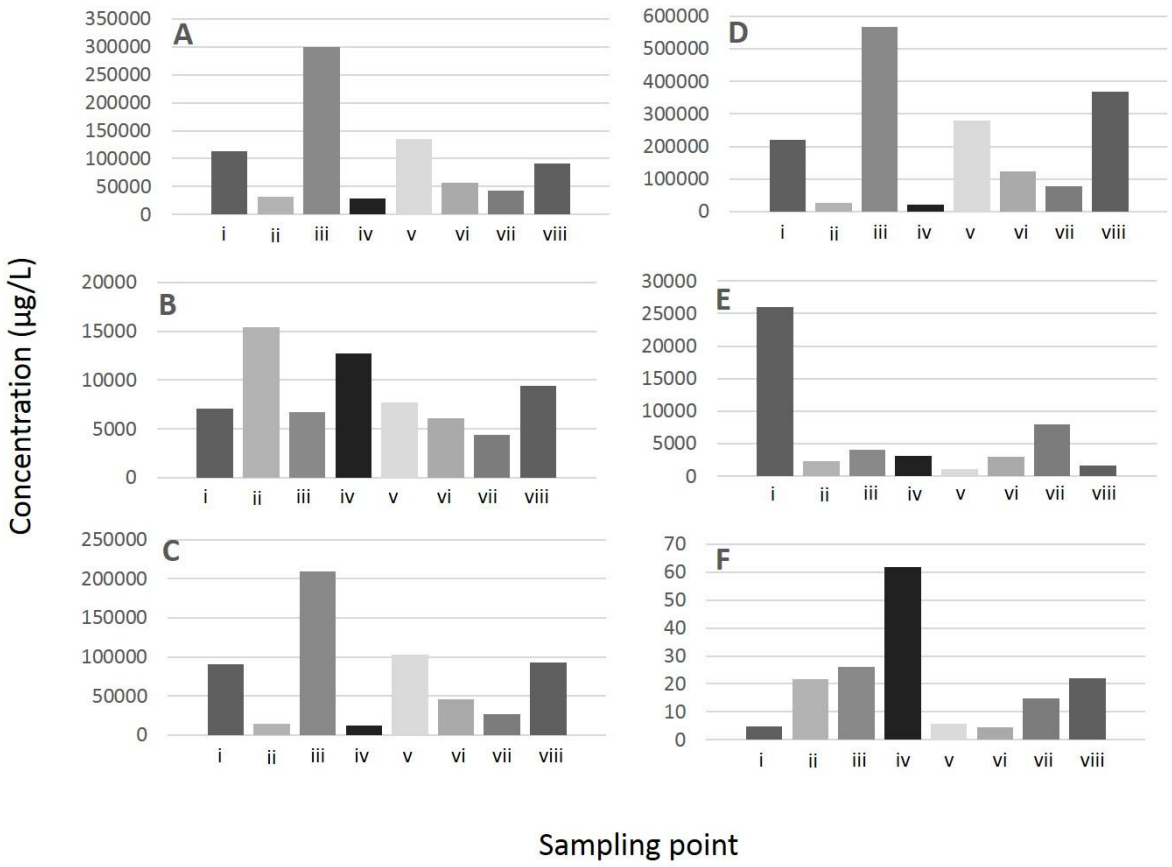


Figure 6.8 Selected volatile compounds (A) 2-methylpropanol, (B) 1-butanol, (C) 2-methyl butanol, (D) 3-methyl butanol, (E) ethyl propanoate, (F) methyl salicylate, detected in laboratory scale distillations of molasses medium fermented using differing starter culture combinations (i) *S. cerevisiae,* (ii) *L. fermentum,* (iii) *L. plantarum,* (iv) *Lactobacillus* spp. (v) *S. cerevisiae & L. fermentum,* (vi) *S. cerevisiae* & *Lactobacillus* spp. (vii) *S. cerevisiae, L. fermentum & Lactobacillus* spp. (viii) Bundaberg Rum

Tables 6.4 and 6.5 show the concentrations of esters and other acetates in the distillates. The main esters found in the distillates were ethyl butanoate, ethyl acetate, ethyl propanoate, ethyl 2-methylpropanoate, ethyl butanoate, ethyl 2-methylbutanoate, ethyl 2-methylbutanoate, ethyl 3-methylbutanoate and ethyl propanoate as also found for the sample of Bundaberg rum. Highest concentrations were produced individually by *S. cerevisiae* and *L. plantarum* with much lesser amounts being produced by either *L. fermentum* or *Lactobacillus* spp. Notably, higher concentrations of ethyl 2-methylpropanoate, ethyl 2-methylbutanoate and methyl salicylate were produced by the lactic acid bacteria than *S. cerevisiae.* The *Lactobacillus* spp. gave three time more methyl salicylate than either *L. fermentum* or *L. plantarum*, (Figure 6.8/Table 6.4). None of the lactic acid bacteria individual fermentations produced ethyl-3-methylbutanoate. The combination of *S. cerevisiae* and *L. fermentum* gave the highest concentrations of most volatiles studied with the exceptions being ethyl-3-methylbutanoate and 2-methyl-propylacetate.

Table 6.4 Esters detected in the distillates of laboratory fermentations of molasses medium with combinations of *Saccharomyces cerevisiae* and species of lactic acid bacteria

Sample	Ethyl Esters (µg/L)							Methyl Ester (µg/L)
Starter culture	Ethyl acetate	Ethyl propanoate	Ethyl 2-methyl propanoate	Ethyl butanoate	Ethyl 2-methyl butanoate	Ethyl 3-methyl butanoate	Ethyl hexanoate	Methyl salicylate[#]
S. cerevisiae	113024	7105	90196	220906	422	28	148	4.8
L. fermentum	31599	15372	14548	25769	2270	-	-	21.6
L. plantarum	300291	6754	209481	566322	7008	-	1039	26.0
***Lactobacillus* spp.**	28162	12706	12432	22453	1747	-	-	61.8
S. cerevisiae* & *L. fermentum	135117	7718	102468	280548[$]	692	15	262	5.7
***S. cerevisiae* & *Lactobacillus* spp**	56371	6106	46204	124944	783	20	115	4.4
***S. cerevisiae*, *L. fermentum* & *Lactobacillus* spp**	42058	4378	26621	79127	507	21	88	14.9
Bundaberg Rum	91552	9382	93009	364888[$]	900	32	123	21.9

-below limit of quantification

$ above calibration limit

methyl salicylate results obtained via method 2 (Chapter 6, Section 6.2.5.4)

Table 6. 5 Other acetates detected in the distillates of laboratory fermentations of molasses medium with combinations of *Saccharomyces cerevisiae* and species of lactic acid bacteria

Sample	Acetates (µg/L)				
Starter culture	2-methyl propyl acetate	2-methyl butyl acetate	3-methyl butyl acetate	2-phenyl ethyl acetate	Hexyl acetate
S. cerevisiae	59	105	545	48	38
L. fermentum	-	10	-	35	-
L. plantarum	91	239	1794	-	19
***Lactobacillus* spp.**	8	12	-	28	-
S. cerevisiae* & *L. fermentum	49	105	932	-	-
***S. cerevisiae* & *Lactobacillus* spp**	25	43	171	-	31
***S. cerevisiae*, *L. fermentum* & *Lactobacillus* spp**	23	31	138	25	28
Bundaberg Rum	5	32	258	-	5

-below limit of quantification

6.4 DISCUSSION

The investigations reported in this Chapter were designed to determine if the species of lactic acid bacteria described in Chapters 3, 4 and 5 impacted on the growth of the starter yeast, *S. cerevisiae*, during fermentation of molasses medium for rum production. As one practical measure of such impact, an aim was to examine key volatiles in distillates of the fermentations. The production of such distillates in sufficient volume necessitated relatively large laboratory scale fermentations (approximately 8 L volumes for each fermentation) and the development of a distillation column and process with sufficient capacity to manage such volumes. These scale up operations brought unforeseen challenges in the preparation of media and starter cultures for the fermentations, management of the fermentations and, finally, the distillation process. In addition, the molasses medium (molasses plus dunder) was a complex matrix that contained unknown factors which, on some occasions, caused unexpected interference with the methods of chemical analyses. Although individual fermentations were conducted in duplicate and various microbiological and chemical analyses were conducted in at least duplicates some variable data were obtained. It is with these limitations that conclusions of the investigations are discussed.

As an individual culture, *S. cerevisiae* fermented the molasses medium with an expected profile - growth to populations of about 10^7 CFU/mL, utilization of sugars (sucrose, glucose and fructose) and production of ethanol. By the end of fermentation, approximately 140 mg/g of fermentable sugars had been utilised giving 5-6% ethanol. However, only about 50 % of the available sugars were utilized, raising the question as to what might be limiting the fermentation and production efficiency. The molasses medium had a high initial content of organic acids (approximates; oxalic acid at 0.3mg/mL; citric acid at 2.0mg/mL; tartaric acid at 3.0 mg/mL; malic acid at 4.0 mg/mL; lactic acid 3.0 mg/mL; acetic acid 2.0 mg/mL; propionic acid at 0.2; butyric acid 9.0 mg/mL; and succinic acid 20.0 mg/mL). This is consistent with previously reported values for molasses (Chapter 2, Table 2.2; Bruijn & Vanis, 1972; Pislor *et al.*, 2009) and dunder, (Chapter 2,

Table 2.4; Chapter 5, Table 5.6; Bories *et al*.,1988) which represented 7.5% of the molasses medium composition. These background levels made it difficult to assess the small changes to their concentrations that might have occurred due to yeast metabolism (Figure 6.5 & Figure 6.6), but the apparent increases in succinic and acetic acids and decrease in malic acid are consistent with *S. cerevisiae* metabolism (Swiegers *et al.,* 2005*)*. The main higher alcohols produced by *S. cerevisiae* from sugar metabolism are 1-propanol, 2-butanol, 1-butanol, 2-methyl butanol, 3-methylbutanol, 2-methylpropanol, amyl alcohol (Timmer, *et al.*, 1971; Suomalainen & Lehtonen, 1979; Swiegers *et al.,* 2005*,* Abbas, 2006). Previous research, summarised in Chapter 2, Table 2.6 lists the important higher alcohols in rum as 1- propanol, 1-butanol, 2-methyl propanol, 2-methylbutanol, 3-methylbutanol (Liebich *et al.,*1970; Lehtonen & Suomalainen, 1977; Sampaio *et al,* 2008). Esters of significance in rum flavour are summarised in Chapter 2, Table 2.6. Of particular importance to the Bundaberg distillery is methyl salicylate which has previously been detected in Jamaican rum (Liebich *et al*, 1970) and a previous study of Bundaberg Rum (Allan, 1972). It is known to give “winter green” sensory notes.

All three species of lactic acid bacteria grew well in the molasses medium, reaching populations over 10^8 CFU/mL which is about 10 fold higher than the maximum population for *S. cerevisiae* (Figure 6.3). Despite such strong growth, these bacteria used little of the fermentable sugars, raising the question as to what they utilize as major growth substrates. As discussed in Chapter 5, these are likely to be the free amino acids that arise from the added dunder. Nevertheless some small amounts of fermentable carbohydrates were utilized and probably accounted for clear increases in the concentration of lactic acid and to a lesser extent acetic acid that was observed in ferments with these bacteria. Such acid production probably accounted for the decrease in pH of the fermentation to about 4.2 during these fermentations. Depending on species and strain, some lactic acid bacteria also utilize organic acids such as malic and citric (Bartowsky & Henschke, 1995; Fleet, 2003) and there was evidence for malic acid utilization by *L. fermentum* possibly by malolactic fermentation. As expected, none of the lactic acid bacteria

produced any significant amounts of ethanol. Analysis of ferments showed, generally lower concentrations of higher alcohols when compared to those produced by *S. cerevisiae*. Hexanol was the exception to this trend, with both *L. fermentum* and *Lactobacillus* spp. producing greater concentrations than the yeast starter culture. Of the higher alcohols analysed in this study, the most prevalent were 2-methylpropanol, 2-methylbutanol and 3-methylbutanol, confirming their presence in rum fermentations. Similar proportions to those from pure *S. cerevisiae* fermentation distillates were seen in the sample of finished Bundaberg Rum product. The concentrations of four key higher alcohols present in Bundaberg rum, when compared to previous research summarised in Chapter 2, Table 2.6, are average to low compared to other rums. 2-methyl propanol and 1- butanol are both within previously reported ranges of rums or fermented molasses. 2-methyl butanol and 3-metyl butanol, however, are both below the previously reported values. The exception is the fermentation of molasses medium by *L. plantarum*, where 2-methyl butanol is within the narrow range reported by Liebich *et al* (1970) and Lehtonen and Suomalainen (1977). 2-methylbutanol sensory characteristics are described as whisky-like or roasted wine.

A small about of the ethyl ester, ethyl acetate, was detected at 48h in the *L. plantarum* fermentation. There is an unusual variation between the higher alcohols detected in the ferment of *L. plantarum* when compared to those detected in the associated distillate. An analytical error during preparation of *L. plantarum* distillate may have been at fault for the higher results reported for the when distillate compared with those obtained from the ferment.

Conclusions about the effect of the lactic acid bacteria on the growth of *S. cerevisiae* may be compromised to some extent by the relatively high initial inoculum of the bacteria relative to *S. cerevisiae* - about 10 fold higher (Figure 6.4). Nevertheless, in all cases the yeast reached maximum populations of about 10^7 CFU/mL by 48 h which is similar to the maximum level when cultured singly. In comparison to the observations during the ecological studies undertaken at the Bundaberg distillery, initial populations of yeast were about 10^7 CFU/mL while

bacterial populations were variable, being between 10^1 – 10^7 CFU/mL. The growth of the lactic acid bacteria was not compromised by the presence of the yeast or the concentrations of ethanol that developed during these laboratory fermentations (Figure 6.4). There was evidence for all three combined fermentations that presence of the bacteria increased sugar fermentation and ethanol production by *S. cerevisiae.* This was more apparent in the *S. cerevisiae* & *Lactobacillus* spp. and the *S. cerevisiae, L. fermentum* and *Lactobacillus* spp. combinations, where final ethanol concentrations were 6.0% and 7.7 % respectively. The reasons for this are not clear but could be due to the lower pH of these fermentation resulting from their production of lactic and acetic acids. As mentioned previously, the molasses medium is a complex matrix that could have inhibitors of yeast metabolism (Kampen, 1975; Lehtonen & Suomalainen, 1977) and it is possible that the bacterial biomass may adsorb or remove such inhibitors. It was mentioned in Chapter 5 that dunder had a stronger inhibitory effect on yeasts than the lactic acid bacteria experienced. Further research is needed to investigate this phenomenon as there is a clear benefit to fermentation efficiency and ethanol production by these mixed fermentations

When compared to the distillate produced from the single culture *S. cerevisiae* ferment, both mixed ferments consisting of *S. cerevisiae* & *Lactobacillus* spp. and *S. cerevisiae, L. fermentum* and *Lactobacillus* spp. produced lower levels of most volatile compounds analysed (Figure 6.3, 6.4, 6.5).The exceptions were the production of hexanol and ethyl 2-methylbutanoate. The fermentation consisting of *S. cerevisiae, L. fermentum* and *Lactobacillus* spp. produced more methyl salicylate when compared to the *S. cerevisiae* only distillates. The esters of ethyl acetate and ethyl butanoate (also known as ethyl n-butyrate) have similar concentrations for some of the laboratory distillates to those seen in previous research (Chapter 2, Table 2.6). The *S. cerevisiae* and *S. cerevisiae & L. fermentum* fermentations, along with the sample of Bundaberg rum were within (or close to) the previously reported ranges. The variation when compared with previously reported results is most likely due to most research on flavour volatiles in rum concentrating on Jamaican based distilleries. Methyl salicylate, specifically

analysed due to its importance to the Bundaberg distillery and possible contribution to the distinctive character of its rum, has an odour threshold of 0.04µg/L (Amerine, *et al.* 1965). The distillates of the *S. cerevisiae* & *L. fermentum* ferments went against this trend displayed by the other mixed ferments and generally produced higher levels of volatiles than the yeast distillates, with a few exceptions, 2-methyl propylacetate, 2-phenyl ethylacetate, hexyl acetate and ethyl 3-methylbutanoate.

Conclusion points

The use of laboratory scale fermentation and distillation apparatus has been used to determine, on a smaller scale, the key elements of microbiological and chemical characteristics of fermentation (Makanjuoula *et al.*, (1992) – whisky). However, there have only been a couple of research groups that have previously investigated rum specific experiments using cane molasses (Arroyo, 1945; and a series of papers by researchers at a “Rum Pilot Plant” at the University of Puerto Rico – Cacho *et al.,* 1986; Cacho & Murphy, 1988).

The type of yeast used for fermentation has a significant effect on the formation of alcohols. *Schizosaccharomyces* pombe, for example, produces relatively little of the higher alcohols (Parfait and Jouret, 1975). The laboratory scale fermentations undertaken during this research did not use a variety of yeasts, only the starter culture obtained from the Bundaberg distillery. The occurrence of co-flocculation, such as those seen in conjunction with brewery yeasts (Zarattini, *et al*, 1993; Domingues *et al*, 2000) may also be occurring in at the distillery.

Project limitations surround distillation which would impact on the results. There is considerable technicality in the art of distillation which was limited in this study due to the size of fermentations used and the physical limitation of the laboratory scale distillation unit. Limitations also exist as there was no study of the impact of ageing/maturation in this research study. There is considerable research into the effects of aging in oak of raw distillates which is known to mellow and also develop some of those flavours (de Torres *et al*., 1987; Mosedale, 1995; Mosedale & Puech, 1998; Singleton, 1995). Other factors such as blending, charcoal filtering

and dilution were also outside of the limitations of this study. The distillates produced in this study, can, however be used as a guide to understand what attributes the finish product can potentially exhibit.

The impact of *L. plantarum* would have been interesting to observe, either individually, in conjunction with *S. cerevisiae* and/or as a more complex mixture with the two other lactic acid bacteria. *Lactobacillus plantarum* was assessed, and excluded, prior to chemical analysis based purely on population levels and frequency of incidence in the ecological surveys performed in Chapters 3 and 4. Perhaps those lower populations were still important for flavour development.

CHAPTER 7 CONCLUSIONS

This thesis describes a systematic investigation of the microbial ecology of molasses fermentation associated with the production of rum at the Bundaberg Distilling Company, Bundaberg, Australia. A combination of cultural and molecular methods was used to isolate, identify and enumerate the microbial species associated with the rum production process. Raw materials and in-process samples from the different stages of the process were examined in surveys conducted during 2006 to 2010. These analyses were followed up with laboratory investigations to gain a deeper understanding of the microbiology of the process.

Chapter 2 examined the background literature on the microbiology and biotechnology of rum production, in general. Although significant advances had been made in describing the chemistry of rum flavour and quality, detailed studies describing the occurrence and growth of microorganisms during molasses based rum fermentations were lacking. While the yeast, *S. cerevisiae* (and to a lesser extent *Schizosaccharomyces pombe*) was mainly responsible for the fermentation various qualitative observations suggested a possible role of bacteria in the process.

Chapter 3 presented an overview of the chain of operations in the production process, along with data obtained from on-site surveys of the microbial ecology of the operation, conducted in 2006, 2008 and 2010. It was concluded from these studies that the yeast *S. cerevisiae* was the predominant microorganism in the fermentations. This was consistent with its preparation and inoculation as a starter culture. In addition to this yeast, lactic acid bacteria were consistently isolated from the fermenting molasses medium and grew to populations of 10^7- 10^8 CFU/mL. The main species found were *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and a *Lactobacillus* spp. These bacteria entered at the stage of propagation. An overview of the cleaning and sanitation operations was undertaken and revealed, while generally efficient, there were some irregularities and oversights. It was concluded that these bacteria probably originated, at some time, as contaminants from the molasses or improperly stored dunder and established residence as endemic, indigenous flora in the myriad of pipework and other areas,

such as heat exchangers, which were difficult to thoroughly clean using CIP programs. Heat exchangers which circulate cooling and heating waters throughout the production chain, appear to be of importance in cross contamination of the endemic microflora from molasses, dunder and contaminated parts of the system to freshly prepared fermentation medium. Further targeted investigation is recommended to determine the precise locations of these contamination points in the production chain. With such information, revised cleaning and sanitation programs and revised quality assurance and control programs could be developed to better manage process microbiology.

Chapter 4 confirmed the endemic association of lactic acid bacteria with the molasses fermentation process by examining the microbial flora in buffer tanks, at the completion of fermentation over an extended period of time (8 months). It confirmed the main species present as *Lactobacillus fermentum, Lactobacillus plantarum, Lactobacillus brevis* and a *Lactobacillus* spp. and that these bacteria occurred at populations of 10^5 – 10^8 CFU/mL, which are quantitatively significant and, therefore, likely to impact on process efficiency and rum product flavour and quality. Fluctuations in the presence and populations of the individual species were observed and would lead to variations in product quality. Such fluctuations confirmed the randomness of the indigenous flora contamination and the need for research to better understand the circumstance that lead to their residence within the distillery. Analyses conducted in this chapter also confirmed the absence of other bacteria (e.g. *Zymomonas, Propionibacterium* and *Clostridium* species) from the fermentations. However, the microbiological analyses conducted in this Chapter as well as those presented in Chapter 3 were based on standard procedures of cultural isolation and may fail to detect the presence of some species. It is recommended that further research and examination of buffer tank samples be conducted with culture independent molecular methods to eliminate the possibility that other bacterial species may also contribute to the molasses fermentations.

Chapter 5 established a greater understanding of dunder, both microbiologically and chemically. Although routine analysis of dunder taken immediately after the still suggested that it was sterile and free of viable microorganisms, it inevitably developed microbial growth on storage. A strain of unidentifiable *Lactobacillus* spp., similar to that isolated from the molasses fermentations reported in Chapters 3 and

4, was consistently isolated from stored dunder. This strain produced extracellular material on heat stress that may confer an advantage of heat tolerance. This may be a novel species and further research is recommended to determine the taxonomic status of this isolate and its physiological characteristics. Chemical analyses of the dunder confirmed its acidic nature and that it was rich in its composition of organic acids and free amino acids. These components, especially the amino acids, may form the substrates for the growth of lactic acid bacteria. However, addition of dunder to the molasses medium did not enhance the growth of the rum fermentation yeast, *S .cerevisiae*, or strains of lactic acid bacteria (*L. fermentum, L. plantarum Lactobacillus* spp.). In contrast, at concentrations of 10% and above, it decreased the growth of these organisms, and would detract from the efficiency of molasses fermentation. The reasons for the use of dunder in molasses based rum fermentations needs to be critically questioned. While the data of this Chapter have advanced knowledge about dunder, it is largely at an exploratory level, and further more detailed research is needed to better understand its microbiological and chemical properties.

Chapter 6 combined the learned knowledge of the microflora of the Bundaberg distillery with small scale laboratory based fermentations and distillations to determine any impact of bacterial contamination on yeast based molasses fermentations. Using the bacterial species isolated in Chapters 3, 4 and 5, this impact was measured by comparing fermentations of bacteria, or bacteria and yeast combinations, with a baseline yeast only fermentation. Cultural and chemical analyses showed that generally lactic acid bacteria had no effect on growth of yeasts and led to more sugar being used by yeast, higher ethanol concentrations and the production of important organic acids, key higher alcohols, esters and acetates. Especially when those higher alcohols, esters and acetates are integral to the flavours of dark rums, such as Bundaberg Rum. The unidentifiable *Lactobacillus* spp. produced the highest concentrations of the desired methyl ester, methyl salicylate, a volatile compound of industrial significance to the Bundaberg distillery.

Finally, the fermentations occurring at the Bundaberg distillery are not pure yeast culture fermentations as the company expected. It is a mixed fermentation of the starter culture yeast and uncontrolled contributions from an indigenous flora of lactic acid bacteria. The lactic acid bacteria probably contribute to the unique and

distinctive flavour of Bundaberg rum, which the Bundaberg Distilling Company is adamant about preserving. It is recommended that the Bundaberg Distilling Company conduct further research to better understand the origin and presence of these lactic acid bacteria and to more precisely determine how the individual species might affect process efficiency and product flavour. Quality assurance and control programs could then be developed to manage the positive or negative impacts of these species. This could also lead to selection of strains of these bacteria for more detailed characterisation and, the possibility of their development as specific starter cultures for use along with *S. cerevisiae* to have greater control over process efficiency and product quality and consistency.

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CHAPTER 9 APPENDICIES

APPENDIX A

Chemical components found in rum

The following table was collated from all available references discussing chemical composition of rum and shows the complexity of rum composition. Concentrations have been given where possible, while some components were only detected qualitatively and not quantitatively. Components with a concentration range were generally detected in more than one study, however results varied; possibly due to different styles of rums tested.

The table has been organised in to chemical groups/families and ordered within each group greatest concentration to least. It is important to note, however, that some components have a greater sensory impact than others regardless of concentration.

Component	Concentration ppm (mg/l)	Component	Concentration ppm (mg/l)
Esters			
Ethyl n-butyrate [a,b,j,k]	220	Isobutyl acetate [a,j,k]	1.5
Ethyl acetate [a,b,j,k]	73-200	Ethyl palmitoleate [a]	1.5
Ethyl n-decanoate [a,i,j,k]	25-130(3.79-103.3)	Ethyl heptenoate [a]	1.2
Ethyl propionate [a,b,j]	50	Ethyl n-hexanoate [a,b,c,i,j,k]	1-40(nd–0.15)
Ethyl linolate [a]	50	Isoamyl n-hexadecanoate [a]	1
Ethyl n-valerate [a,b,j,k]	40	β-phenylethyl acetate [a,k]	1
Ethyl n-octanoate [a,b,i,j,k]	15-50(0.65-14.14)	n-hexyl acetate [a,b]	1
Isoamyl n-ocatnoate [a]	15	Ethyl n-pentadecanoate [a]	0.8
Ethyl n-dodecanoate [a,i,j]	12-15(0.03-1.59)	Diethyl succinate [a,b,k]	0.8
Ethyl lactate [a,b,k]	10	Ethyl benzoate [a,b,k]	0.5
Ethyl formate [a,b,j, k]	10	n-propyl acetate [a,b,j]	0.5
Ethyl isobutyrate [a,b,k]	8	Ethyl sterate [a]	0.5
Ethyl oleate [a]	8	Isoamyl n-valerate [a]	0.5
Ethyl n-hexadecanoate [a,j]	5-50	Isobutyl propionate [a,j]	0.5
Isoamyl acetate [a,j]	5-10	Ethyl (β-phenylpropionate) [a]	0.5
Ethyl n-tetradecanoate [a,j]	5-6	Methyl n-hexandecanoate [a]	0.3
Ethyl n-heptanoate [a,b]	2.5	Ethyl n-nonanoate [a,b]	0.3
Ethyl isovalerate [a,k]	2.5	Ethyl (3-methoxy-4-hydroxybenzoate) [a,k]	0.3
Ethyl (2-methylbutyrate) [a]	2.5	Methyl n-decanoate [a,d]	0.2
Monoethyl succinate [a]	2	n-propyl propionate [a]	0.2
Isoamyl formate [a]	2	Isoamyl n-tetradecanoate [a]	0.2
Isoamyl n-butyrate [a,j]	1.5	Isoamyl n-decanoate [a,j]	0.2

Component	Concentration ppm (mg/l)	Component	Concentration ppm (mg/l)
Esters (cont.)		**Alcohols (cont.)**	
β-phenylethyl n-decanoate [a]	0.2	2-hexanol [a]	0.02
Methyl salicylate [a,j,k]	0.15-25	Methyl butenol [d]	-
Isobutyl n-decanoate [a]	0.15	2-methyl-1-propanol [d,j]	-
An ethyl dodecanoate [a]	0.15	4-methylpentan-1-ol [k]	-
An ethyl dodecanoate [a]	0.13	3-methyl-2-buten-1-ol [k]	-
Isoamyl n-dodecanoate [a,j]	0.13	2-heptanol [k]	-
Ethyl n-undecanoate [a]	0.13	(Z)-4-hepten-2-ol [k]	-
Isoamyl propionate [a]	0.1	(z)-3-hexen-1-ol [k]	-
Methyl n-octanoate [a]	0.1	2-octanol [k]	-
β-phenylethyl n-octanoate [a]	0.1	1-heptanol [k]	
Ethyl (2-furancarboxylate) [a]	0.1	1-octen-3-ol [k]	
Methyl n-dodecanoate [a]	0.1	1-octanol [k]	
n-propyl n-hexadecanoate [a]	0.05	2-nonanol [k]	
Ethyl n-heptadecanoate [a]	0.05	(z)-2-octen-1-ol [k]	
n-propyl n-butyrate [a,j]	0.03	1-decanol [k]	
n-butyl acetate [a,b,j]	0.01	**Phenols**	
ethyl 2-ethoxyacetate [k]	-	Isoeugenol [a]	0.08
Ethyl (3-methylbutyrate) [b]	-	4-methyl-2-methoxyphenol [a,k]	0.05
3-methylbutyl acetate [b,k]	-	4-ethyl-2-methoxyphenol [a,k]	0.02
Ethyl-3-hydroxybutanoate [b,k]	-	4-ethylphenol [a,e,f,k]	nd-1.96
Isobutyl valerate [b]	-	2 ethyl-2-methoxy-phenol [e,f]	nd-1.83
3-methylbutyl butanoate [b]	-	Eugenol [a,b,e,f,j,k]	nd-1.36
Ethyl nonadecanoate [d]	-	2-methoxy-4-propylphenol [e,k]	nd-0.84
Diethyl carbonate [k]	-	2-methoxyphenol [a,e,f,k]	nd-0.83
Ethyl 3-ethoxypropanoate [k]	-	Phenol [a,e,f,k]	tr-0.26
Alcohols		4 methylphenol [e,f]	nd-0.2
Ethanol [a,b,k]	(see below)	2 methylphenol [e,f]	nd-0.1
3-methyl-1-butanol [a,b,d,j,k]	860-1000	3 methylphenol [e]	nd-0.05
2-methyl-1-butanol [a,b,d,j]	200-210	2-methoxy-4-methylphenol [b,f]	-
Isobutanol [a,b,k]	100	3-methyl-3-buten-1-ol [k]	-
n-butanol [a,b,j,k]	10	**Lactones**	
n-propanol [a,b,d,j,k]	7.5-420	δ-octalactone [a]	0.1
2-butanol [a,b,k]	6	ɣ-nonalactone [a,k]	0.025
1-hexanol [a,j,k]	1.3-2.5	ɣ-decalactone [a]	0.025
1-pentanol [a,b,j]	1-2.5	δ-decalactone [a]	0.025
β-phenylethanol [a,j,k]	0.5-<1.0	ɣ-dodecalactone [a]	0.025
Menthol [a]	0.5	δ-dodecalactone [a]	0.025
2-pentanol [a,k]	0.5	**Carbonyl compounds**	
2-methyl-2-butanol [a]	0.2	Furfural [a,b,k]	25
3-pentanol [a,k]	0.13	4-ethoxy-2-pentanone [a,k]	7.5
Phenylmethanol [j,k]	<0.1	3-penten-2-one [a,k]	7
Methanol [a]	0.08	4-ethoxy-2-butanone [a]	5

Component	Concentration ppm (mg/l)	Component	Concentration ppm (mg/l)
Carbonyl compounds (cont.)		**Acetals (cont.)**	
2-methyl-3-tetrahydrofuranone [a,k]	5	1-ethoxy-1-(2-methylpropoxy)-ethane [a]	1.5
Benzaldehyde [a,b,k]	2	1,1-di-(2-methylpropoxy)-propane [a]	1.3
Isovaleraldehyde [a,b,k]	1.8	1,1-diethoxypropane [a]	1.2
2-methylbutyraldehyde [a]	1.5	1-ethoxy-1-propoxyethane [a]	1.2
5-methylfurfural [a,b,k]	1.2	1-ethoxy-1-propoxy-2-methylpropane [a]	1
2-pentanone [a,k]	1.2	1-(2-methylpropoxy)-1-(3-methylbutoxy)-2-methylpropane [a]	0.8
Acetylfuran [a,b]	1.2		
Acetaldehyde [a,b,j]	0.5-150	1,1-di-(2-methylpropoxy)-3-methylbutane [a]	0.8
Isobutyraldehyde [a]	0.25		
Vanillin [a,k]	0.25	Diethoxymethane [a]	0.8
Acetone [a,k]	0.25	1,1-di-(2-methylpropoxy)-2-methylpropane [a]	0.5
2-butanone [a,k]	0.03		
Diacetyl [a,k]	0.03	1-ethoxy-1-(3-methylbutoxy)-propane [a]	0.5
o-hydroxyacetonphenone [a]	0.01	1-ethoxy-1-butoxyethane [a]	0.5
Propionaldehyde [a,b]	0.01	1-ethoxy-1-pentoxyethane [a]	0.5
but-2-enal [b]	-	1-butoxy-1-(3-methylbutoxy)-ethane [a]	0.5
Hexanal [b]	-	1-methoxy-1-ethoxyethane [a]	0.3
1,1,3 triethoxypropane [k]	-	1-ethoxy-1-(2-methylpropoxy)-propane [a]	0.25
Prop-2-enal [b]	-	1,1-dipropoxyethane [a]	0.25
2-ethoxypropanal [b]	-	1-ethoxy-1-(2-methylpropoxy)-pentane [a]	0.25
Acetals		1-propoxy-1-(3-methylpropoxy)-3-methylbutane [a]	0.25
1,1-diethoxyethane [a,b,j,k]	50-320		
1,1-diethoxy-3-methylbutane [a, k]	13	1-(2-methylpropxy)-1-(3-methylbutoxy)-3-methylbutanse [a]	0.25
1-ethoxy-1-(3-methylbutoxy)-ethane [a]	10		
1,1-di-(3-methylbutoxy)-ethane [a]	7.5	1,1-di-(3-methylbutoxy)-2-methylpropane [a]	0.12
1,1-diethyoxy-2-methylpropane [a]	6		
1-(2-methylpropoxy)-1-(3-methylbutoxy)-ethane [a]	5	1-ethoxy-1-(2-methylpropoxy)-2-methylpropane [a]	0.12
1-ethoxy-1-(3-methylbutoxy)-2-methylpropane [a]	5	1-propoxy-1-(3-methylbutoxy)2-methylpropane [a]	0.12
1-ethoxy-1-(2-methylpropoxy)-3-methylbutane [a]	5	1-propoxy-1-(2-methylpropoxy)-2-methylpropane [a]	0.12
1-ethoxy-1-(3-methylbutoxy)-3-methylbutane [a]	4	1-propoxy-1-(3-methylbutoxy)-propane [a]	0.12
		1,1-dipropoxy-3-methylbutane [a]	0.12
1,1-diethoxy-2-methylbutane [a]	2.5	1,1-di-(3-methylbutoxy)-3-methylbutane [a]	0.12
1-ethoxy-1-(2-methylbutoxy)-ethane [a]	2.5	1-propoxy-1-(2-methylpropoxy)-3-methylbutane	0.08
1-propoxy-1-(3-methylbutoxy)-ethane [a]	2.5		
1-(3-methylbutoxy)-1-(2-methylbutoxy)-ethane [a]	2.3	1,1-di-(2-methylpropoxy)-pentane [a]	0.05
		1-ethoxypropane [k]	-
1,1-diethoxy-2-propanone [a]	1.5	1,1-ethoxy-hexoxyethane [b]	-
1,1-di-(2-methylpropoxy)-ethane [a]	1.5	1-1 diethoxymethane [k]	-

Component	Concentration ppm (mg/l)	Component	Concentration ppm (mg/l)
Acids		**Hydrocarbons**	
Acetic acid [a,b,d,j,k]	10.3-35	3,8,8-trimethyl-tetrahydronaphthalene [a]	5
n-octanoic acid [a,b,c,j,k]	4.3-7.5 (8.9-24.1)	3,8,8-trimethyl-tetrahydronaphthalene [a]	1.2
Dodecanoic [c,j,k]	4.0(6.5-12.0)	3,8,8-trimethyl-dihyrdronaphthalene [a]	0.25
hexadecanoic acid [c,j]	1.4(0.5-4.0)	n-pentane [a]	0.05
Tetradecanoic acid [c,j]	0.7(0.4-1.7)	2-methylpentane [a]	0.01
Isovaleric acid [a,j,k]	0.7 (3.0-6.5)	n-hexane [a]	0.05
n-decanoic acid [a,c,j,k]	0.6-13.4	n-heptane [a]	0.05
2-methylbutyric acid [a,k]	0.6	2-methylhexane [a]	0.025
Heptenoic acid [a,]	0.5	2,3-dimethylpentane [a]	0.025
2-furancarboxylic acid [a]	0.4	Methylcyclohexane [a]	0.003
Benzoic acid [a]	0.4	**Nitrogen compounds**	
n-hexanoic acid [a,b,c,j,k]	0.3-15 (4.5-6.6)	Pyridine [g]	-
n-butyric acid [a,b,j,k]	0.3-7.5 (8.0-15.3)	2-methylpyridine [g]	-
3-furancarboxylic acid [a]	0.3	1,3-thiazol [g]	-
4-methyl pentanoic [j]	0.3	Methylpyrazine [g]	-
n-heptanoic acid [a,c,j]	0.28-2.5 (tr-2.4)	3-methylpyridine [g]	-
Propionic acid [a,b,c,k]	0.2-1.5(7.4-30.2)	2,5-dimethylpyrazine [g]	-
Isobutyric acid [a,b,j,k]	0.2-0.9 (2.9-4.3)	2,6-dimethylpyrazine [g]	-
β-ethoxypropionic acid [a]	0.15	2,3-dimethylpyrazine [g]	-
Isohexanoic acid [a]	0.09	2-methyl-6-ethylpyrazine [g]	-
Octadecanoic acid [c,j]	0.08(0.2-0.5)	2-methyl-5-ethylpyrazine [g]	-
n-nonanoic acid [a,c]	0.06 (tr-0.5)	Trimethylpyrazine [g]	-
n-valeric acid [a,b,j]	0.04-7.5 (1.5-6.5)	2,5-dimethyl-3-ethylpyrazine [g]	-
Isooctanoic acid [a]	0.03	**Miscellaneous**	
Oleic acid [c]	(0.2-1.2)	α-ionone [a*]	0.3
Hexadec-9-enoic acid [c]	(0.2-1.0)	Unsaturated decalactone [a*]	0.2
Linoleic acid [c]	(nd-2.2)	Dimethyl sulphide [a,j]	0.12
Undecanoic acid [c]	(nd-0.7)	Methylethyl sulphide [a]	0.12
Pentadecanoic acid [c]	(nd-0.1)	sec. butyl acetate [a*]	0.1
Tridecanoic acid [c]	(nd-0.1)	isobutyl n-butyrate [a*]	0.1
2-ethyl-3-methylbutyric acid [h]	-	methylbutenol [a*]	0.1
Butanoic acid [k]	-	Diethyl ether [a]	0.05
Alkylpyrazines		2-methylfuran [a]	0.05
2,6-dimethylpyrazine [a]	1.5	Methyl acetate [a*]	0.025
2,5-dimethylpyrazine [a]	0.8	4-methyl-3-pentene-2-one [a*]	0.025
2-methylpyrazine [a]	0.5	2-hexanone [a*]	0.025
2-methyl-3-ethylpyrazine [a]	0.25	A hydroxymethylbenzaldehyde [a*]	0.01
2,5-dimethyl-3-ethylpyrazine [a]	0.2	3-hexanone [a*]	0.005
3,5-dimethyl-2-ethylpyrazine [a]	0.1	Ethyl dodecadienoate [a*]	0.03
2-methyl-6-ethylpyrazine [a]	0.1	Isobutyl formate [a*]	0.025

Note: Ethanol concentration depended on sampling point etc. Pino data included samples containing 80% ethanol v/v.

[a] Liebich *et al* (1970) [*]tentatively identified in this work, [b] Maarse and ten Noever de Brauw (1966), [c] Nykanen *et al* (1968), [d] Gracia *et al* (2007), [e] Lehtonen (1983), [f] Timmer *et al* (1971), [g] Wobben *et al* (1971), [h] Lehtonen *et al* (1977), [i] Pino *et al* (2002), [j] Allan (1972), [k] Pino *et al* (2012) (-) denotes identification made however no quantification performed.

APPENDIX B

Individual culture fermentations

S. cerevisiae

	Time Station	Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
						Fructose	Glucose	Sucrose
1	0	5.5	5.3	31.3	0.0	54.0	63.5	123.5
	8	5.4*	5.3	31.4	0.3	51.8	48.4	135.0
	16	6.0	5.3	31.4	0.4	46.7	47.1	136.7
	24	6.4	5.3	30.0	2.3	42.3	66.9*	108.4
	32	6.6	5.3	28.6	3.6	37.7	43.9	74.3
	40	6.9	5.2	28.8	3.6	37.2	39.7	61.0
	48	6.8	5.2	27.2	5.5	28.1	34.9	55.9
2	0	6.1	5.3	31.2	0.0	53.8	61.1	125.9
	8	5.2*	5.3	31.1	0.3	37.0	66.1	121.2
	16	6.0	5.2	31.0	0.7	28.6	55.7	158.3*
	24	6.3	5.2	29.3	2.8	30.8	55.3	115.0
	32	6.5	5.2	27.9	4.1	26.1	48.1	58.2
	40	7.1	5.2	27.3	4.4	25.6	44.7	52.9
	48	6.8	5.2	26.3	4.6	25.5	41.5	51.5
Average	0	5.8	5.3	31.3	0.0	53.9	62.3	126.6
	8	5.3*	5.3	31.3	0.3	44.4	57.3	128.1
	16	6.0	5.3	31.2	0.6	37.6	51.4	136.7
	24	6.4	5.3	29.7	2.6	36.6	55.3	111.7
	32	6.6	5.3	28.3	3.9	31.9	46.0	66.3
	40	7.0	5.2	28.1	4.0	31.4	42.2	57.0
	48	6.8	5.2	26.8	5.1	26.8	38.2	53.7

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

L. fermentum

	Time Station	Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
						Fructose	Glucose	Sucrose
1	0	6.9	5.3	30.4	0.0	47.5	74.2	141.6
	8	6.9	5.2	30.2	0.2	47.9	73.9	137.2
	16	7.6	5.2	29.7	0.3	45.7	73.2	138.6
	24	8.1	4.9	29.5	0.3	42.0	73.6	145.1*
	32	8.4	4.5	29.8	0.3	48.8	72.6	131.5
	40	8.3	4.3	29.5	0.3	41.5	86.8*	122.5
	48	8.2	4.2	29.4	0.3	42.3	77.7	122.5
2	0	7.0	5.3	30.3	0.0	56.1	79.2	115.6
	8	7.2	5.3	30.1	0.2	59.1	74.8	109.2
	16	7.4	5.1	29.6	0.2	56.5	73.8	103.7
	24	8.4	4.9	29.3	0.3	50.1	72.4	136.5
	32	8.2	4.6	29.6	0.3	45.4	83.8*	126.4
	40	8.4	4.3	30.0	0.3	44.3	87.0*	115.8
	48	8.3	4.1	28.9	0.3	43.4	77.9	104.4
Average	0	7.0	5.3	30.4	0.0	51.8	76.7	128.6
	8	7.1	5.3	30.2	0.2	53.5*	74.4	123.2
	16	7.5	5.2	29.7	0.3	51.1	73.5	121.2
	24	8.3	4.9	29.4	0.3	46.1	73.0	136.5*
	32	8.3	4.6	29.7*	0.3	47.1	72.6	129.0
	40	8.4	4.3	29.8*	0.3	42.9	86.9*	119.2
	48	8.3	4.2	29.2	0.3	42.9	77.8	113.5

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

L. plantarum

	Time Station	Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
						Fructose	Glucose	Sucrose
1	0	6.0	5.1	30.2	0.0	52.8	76.6	122.6
	8	6.0	5.1	30.1	0.2	72.0*	45.5	116.5
	16	6.9	5.0	30.0	0.1	47.5	47.6	119.1
	24	7.7	4.8	30.2	0.2	40.1	52.1	123.4
	32	8.4	4.6	30.1	0.2	40.1	48.2	110.9
	40	8.2	4.4	29.9	0.2	37.1	48.3	131.0*
	48	8.6	4.4	29.8	0.2	36.6	45.3	140.8*
2	0	6.6	5.1	31.2	0.0	58.8	82.8	132.8
	8	6.5	5.1	31.1	0.1	94.6*	49.1	129.7
	16	8.1	5.0	31.0	0.2	60.7	47.3	101.9*
	24	8.0	4.8	30.6	0.1	43.3	47.0	114.0
	32	8.7	4.6	30.7	0.2	41.4	48.0	103.1
	40	8.7	4.4	30.3	0.2	41.1	43.9	111.5*
	48	8.6	4.4	30.0	0.2	42.2	40.9	128.4*
Average	0	6.3	5.1	30.7	0.0	55.8	79.7	127.7
	8	6.2	5.1	30.6	0.2	83.3*	47.3	123.1
	16	7.5	5.0	30.5	0.1	54.1	47.5	119.1
	24	7.8	4.8	30.4	0.2	41.7	49.6	118.7
	32	8.5	4.6	30.4	0.1	40.8	48.1	107.0
	40	8.4	4.4	30.1	0.2	39.1	46.1	121.3*
	48	8.6	4.4	29.9	0.2	39.4	43.1	134.6*

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

Lactobacillus spp.

	Time Station	Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
						Fructose	Glucose	Sucrose
1	0	6.5	5.3	31.1	0.0	53.7	82.1	124.5
	8	7.2	5.3	30.9	0.2	65.1*	85.4	110.8
	16	7.5	5.1	31.4	0.2	56.6	85.5	120.5
	24	7.6	4.7	30.7	0.3	34.1*	91.4*	123.6
	32	8.3	4.4	30.2	0.3	51.0	88.0	118.3
	40	8.2	4.3	30.6	0.3	45.2	83.1	118.6
	48	7.9	4.2	30.2	0.2	42.6*	88.6*	127.9
2	0	7.3	5.3	31.6	0.1	55.5	81.4	130.3
	8	7.2	5.3	31.1	0.2	55.8	84.7	109.3
	16	7.8	5.2	31.0	0.2	55.5	94.1*	136.6
	24	7.9	4.6	29.3	0.2	49.1	90.7*	131.5
	32	8.1	4.4	27.9	0.2	50.6	83.1	132.9
	40	8.2	4.3	27.3	0.2	41.8	83.0	125.2
	48	8.9	4.1	26.3	0.2	36.5	78.2	135.5*
Average	0	6.9	5.3	31.3	0.1	54.6	81.8	127.4
	8	7.2	5.3	31.0	0.2	55.8	85.1	110.1*
	16	7.7	5.2	31.2	0.2	56.1	85.5	128.6
	24	7.7	4.7	30.0	0.3	49.1	91.1*	127.6
	32	8.2	4.4	29.1	0.3	50.8	85.6	125.6
	40	8.2	4.3	29.0	0.3	43.5	83.1	121.9
	48	8.4	4.2	28.3	0.2	39.6	78.2	127.9

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

Mixed culture fermentations

S. cerevisiae & *L. fermentum*

	Time Station	Bacteria Log CFU/mL	Yeast Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
							Fructose	Glucose	Sucrose
1	0	7.3	5.0	5.2	30.4	0.1	54.4	62.9	137.4
	8	7.4	5.3	5.1	29.1	0.7	26.3	59.3	102.3*
	16	7.6	6.0	5.0	28.5	0.8	20.4	57.3	170.6*
	24	7.7	6.8	4.9	26.7	2.0*	22.8*	48.5	166.7*
	36	8.0	7.1	4.8	24.6	5.6	15.9	21.5	113.7
	48	8.0	7.2	4.6	23.2	7.4	5.1	18.8	110.7
2	0	7.4	7.4*	5.2	30.6	0.1	51.6	79.4	134.7
	8	7.1	5.8	5.1	30.1	0.6	41.9	45.0	139.5
	16	7.5	6.0	5.0	29.6	0.8	62.3*	79.7*	140.3
	24	7.4	6.6	4.9	27.8	0.3*	21.9	54.3	129.9
	36	7.7	7.1	4.8	25.4	5.3	11.6	35.4	139.7*
	48	7.9	7.2	4.7	23.6	8.4	7.9	27.0	108.1
Averages	0	7.3	5.0	5.2	30.5	0.1	53.0	71.1	136.1
	8	7.3	5.6	5.1	29.6	0.7	34.2	52.2*	139.5
	16	7.6	6.0	5.0	29.1	0.8	20.4	57.3	140.3
	24	7.6	6.7	4.9	27.3	2.0	21.9	51.5	129.9
	36	7.9	7.1	4.8	25.0	5.5	13.8	28.5	113.7
	48	8.0	7.2	4.7	23.4	7.9	6.6	24.5	109.4

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

S. cerevisiae & *Lactobacillus* spp.

	Time Station	Bacteria Log CFU/mL	Yeast Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
							Fructose	Glucose	Sucrose
1	0	7.4	5.9	5.2	31.9	0.1	42.5	66.6	180.8
	8	7.3	5.1	5.2	31.4	0.4	35.4	63.2	137.2
	16	7.6	5.7	5.2	31.2	0.2	37.5	45.6	110.8
	24	7.2	5.6	5.0	28.1	0.9	46.0*	36.2	104.3
	36	8.7	6.7	4.5	28.2	5.7	30.5	33.7	81.8
	48	8.6	7.1	4.3	27.9	6.0	26.2	29.4	81.4
2	0	7.1	5.8	5.2	31.3	0.1	58.3	52.5	102.3
	8	7.5	4.7	5.2	31.1	0.4	46.8	53.9	142.0
	16	7.5	5.7	5.2	31.0	0.2	37.8	46.5	122.7
	24	8.1	6.2	5.0	29.7	0.8	38.1	40.5	86.1
	36	8.5	6.9	4.5	27.8	6.7*	45.5*	35.3	89.3
	48	8.5	7.1	4.6	27.4	13.2*	35.2*	31.7	127.3*
Averages	0	7.3	5.9	5.2	31.6	0.1	50.4	59.6	141.6
	8	7.4	4.9*	5.2	31.3	0.4	41.1	58.6	139.6
	16	7.6	5.7	5.1	31.1	0.2*	37.7	46.1	116.8
	24	7.8	5.8	5.0	28.9	0.9	38.1	38.4	95.2
	36	8.6	6.8	4.5	28.0	6.2	30.5	34.5	85.6
	48	8.6	7.1	4.5	27.7	6.0	26.2	30.6	81.4

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

S. cerevisiae, *L. fermentum* & *Lactobacillus* spp.

	Time Station	Bacteria Log CFU/mL	Yeast Log CFU/mL	pH	°Brix	Ethanol (%)	Sugars (mg/g)		
							Fructose	Glucose	Sucrose
1	0	7.2	5.9	5.2	27.5	0.1	42.4	73.2	148.3
	8	7.1	5.9	5.2	27.6	0.3	38.7	48.4*	72.6*
	16	7.9	5.8	5.0	23.9	1.2	36.8	64.4	77.4
	24	8.4	6.5	4.5	26.1	0.8*	47.1*	24.4	58.9
	36	8.3	7.0	4.3	23.1	3.7	37.8*	27.6	65.3
	48	8.6	7.7	4.1	22.2	7.7	24.5	24.4	63.8
2	0	7.2	5.5	5.2	29.5	0.1	37.9*	73.3	17.9*
	8	7.4	5.6	5.1	29.8	0.3	34.0	73.2	81.3
	16	7.6	6.1	4.9	29.3	0.8	32.7	72.4	80.2
	24	8.3	6.5	4.5	25.8	0.6*	30.6	62.6	79.1
	36	8.6	7.3	4.4	27.4	4.5	30.0	61.4	80.0*
	48	8.7	7.8	4.2	26.2	13.7*	28.4	51.6	66.5*
Averages	0	7.2	5.7	5.2	29.5	0.1	42.4	73.3	148.3
	8	7.3	5.8	5.1	28.7	0.3	36.4	73.2	81.3
	16	7.8	6.0	5.0	26.6	1.0	34.8	68.4	78.8
	24	8.4	6.5	4.5	26.0	0.7*	30.6	43.6	69.0
	36	8.5	7.2	4.4	25.3	4.1	30.0	44.6	65.3
	48	8.7	7.8	4.2	24.2	7.7	26.5	38.0	63.8

*indicates the results identified as outliers and are excluded from graphical representations discussed in Chapter 6.

APPENDIX C – ORGANIC ACIDS

Raw data with notations (*) on results that had significant changes (p<0.05) after a one way single factor analysis of variance and t-test were performed to determine significant differences between means.

Individual culture fermentations (results expressed as averages of duplicate fermentations)

S. cerevisiae

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	Total
0	5.32	0.21	0.82	3.44	6.85	2.92	1.80	0.23	9.37	20.03	45.67
8	5.32	0.21	0.60	3.74	7.07*	2.49	1.95	0.20*	9.33	18.26	43.85
16	5.26	0.19*	0.66*	3.64*	6.97	2.56*	1.75*	0.19*	9.03	17.91	42.90
24	5.25	0.22	0.90	4.15	6.85	3.17*	2.23	0.23*	11.86*	25.64*	55.25
32	5.26	0.23*	0.83	3.91*	5.68	3.10*	2.34	0.27	13.12*	24.27*	53.75
40	5.17	0.24	0.82	3.75	5.95	2.94	2.50*	0.25*	10.82	21.88	49.15
48	5.18	0.21	0.76	3.52*	5.54*	2.75	2.20*	0.23*	11.46	21.81	48.48

L. fermentum

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	Total
0	5.25	0.34	0.88	4.16	8.10	2.97	1.95	0.20	10.14	20.81	49.55
8	5.22	0.36	1.18	4.77	8.93*	3.21	1.85	0.56	8.09	18.37	47.32
16	5.17	0.20	1.32	5.06	4.57	3.24	2.40*	0.09*	9.06	17.98	43.92
24	4.89	0.34	1.06	3.89	3.94*	4.58*	1.64	0.19*	10.15*	16.11*	41.90
32	4.54	0.38	1.20	3.91	3.46*	4.49*	1.83	0.85	11.75*	17.39	45.26
40	4.30	0.42*	1.37	4.49	5.93	3.47	1.85	0.60	10.87	18.90	47.90
48	4.15	0.43*	1.34	3.85	5.32	3.90	2.31	0.70	11.43	18.45	47.73

Individual culture fermentations (cont.)

L. plantarum

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	Total
0	5.10	0.27	1.07	3.87	4.30	3.00	1.94	0.12	6.97	20.25	41.79
8	5.09	0.27	1.02*	3.82*	4.51	3.44	1.80	0.09	6.03	24.12*	45.10
16	5.01	1.39	1.72	3.18	3.82	5.20*	1.14	0.06	5.87	22.39	44.77
24	4.81	1.31*	1.59	3.10	3.53*	6.81*	1.03*	0.17	2.86	20.68*	41.08
32	4.55	1.33*	1.64	3.00	7.26	6.07*	1.50	0.17	3.51	21.30	45.78
40	4.41	1.28*	1.71	2.50	7.82	5.20	2.89*	0.07	4.86	19.41	45.74
48	4.35	1.19	1.49	2.10	8.67*	7.37	2.99	0.12	6.14	17.65*	47.72

Lactobacillus spp.

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	total
0	5.30	0.30	1.07	4.29	5.00	3.08	2.07	0.12	10.40	22.78	49.11
8	5.27	0.32	0.87*	4.82*	9.35	4.80	3.96	0.26	13.89	22.60	60.87
16	5.16	0.25*	0.84*	4.45	9.38*	4.50	3.70	0.23	13.15*	22.56*	59.06
24	4.65	0.29	1.91	4.49	6.01	4.00	4.13*	0.42	12.55	20.48*	54.28
32	4.41	0.30	0.95	4.50	8.13*	5.80	4.11	0.16*	11.06	18.35	53.36
40	4.26	0.31	1.63	4.12*	4.33	6.10*	5.07*	0.32	5.67*	18.11	45.66
48	4.16	0.33*	1.02	4.12	6.45	6.70*	4.15	0.07*	6.51*	16.39*	45.74

Mixed culture fermentations

S. cerevisiae & *L. fermentum*

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	total
0	5.08	0.30	2.07	2.91	2.54	3.97	2.16	0.30	9.06	21.57	44.88
8	5.06	0.30	2.20	2.98	2.51	5.63	2.25*	0.30	8.51	22.25*	46.93
16	5.01	0.29	2.20	2.99	2.42	5.25*	2.21*	0.44	9.77	24.02	49.59
24	4.92	0.39	1.71*	2.73	1.05*	6.26*	1.40	0.96	9.86	23.12	47.48
36	4.79	0.32	2.30	3.15	1.88	6.41*	2.03	0.61	11.45	29.91*	58.06
48	4.68	0.30	2.35*	3.22	9.47*	6.63*	2.00	0.41	10.07	31.44*	65.89

S. cerevisiae & *Lactobacillus* spp.

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	total
0	5.18	0.33	2.56	3.37	1.52	3.21	2.37	0.22*	10.47	19.85	43.90
8	5.18	0.33	2.58	3.40	1.34	6.65*	2.25	0.24	10.33*	24.48	51.60
16	5.18	0.32	2.69*	3.54	1.76*	7.32	2.38	0.24	11.23	25.07	54.55
24	4.98	0.33	2.60	3.35	1.40	7.42	2.11*	0.25	10.85	27.08	55.39
36	4.49	0.33	2.55	3.44	1.14*	8.86*	2.16	0.34	12.64	26.10	57.56
48	4.40	0.32	2.69	3.56	1.17	12.62*	2.56*	0.43	15.66*	30.60*	69.61

Mixed culture fermentations (cont.)

S. cerevisiae, *L. fermentum* & *Lactobacillus* spp.

Time Station	pH	Organic Acids									
		Oxalic	Citric	Tartaric	Malic	Lactic	Acetic	Propionic	Butyric	Succinic	total
0	5.21	0.22	1.49	2.02	1.63	3.72*	1.79	0.31	9.80*	20.41	41.39
8	5.15	0.18	1.35	1.87	2.26*	5.07	1.75	0.25	7.61*	20.44*	40.78
16	4.95	0.28	1.74	2.03	1.86	5.62	1.97	0.27	9.17	36.20	59.14
24	4.52	0.24	1.24	1.76	3.27	5.92	1.97	0.32	8.58*	50.14*	73.44
36	4.33	0.12	1.41	1.93	2.66	5.69	1.74	0.43	10.86*	43.11	67.95
48	4.16	0.11	1.41	1.98	2.27	9.18*	1.37*	0.54	13.96*	31.09*	61.91