

African mead: biotechnology and indigenous knowledge systems in *iQhilika* process development

by

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This thesis is dedicated to the memory of Conrad Cambray, my brother, who would have enjoyed seeing this work completed.

Abstract

While the production of mead, a fermented honey beverage, has declined in popularity around the world in recent centuries, a substantial mead industry continues to exist in Africa with an estimated annual production of 1 to 1.7 billion litres. This is largely an 'invisible industry', and has functioned outside the formal economy due to proscription of indigenous beverages during colonial times. The traditional African mead industry is, however, also now under pressure due to the environmental degradation of scarce natural ingredients, urbanisation and loss of indigenous knowledge systems (IKS) and, with time, the beverage will likely follow the declining trend of mead consumption observed elsewhere. An analysis of early reports of African mead production suggested that the Khoi-San, among the earliest inhabitants of the continent, are the originators of the mead making techniques which use fibrous plant materials derived from specific plant species, to facilitate mead fermentation in some way.

The Eastern Cape represents a region with a large body of Khoi-San IKS preserved in their descendants among the Afrikaans and Xhosa populations. A survey to establish a baseline of mead-making technology in the Eastern Cape was undertaken, and involved interviewing traditional mead makers across an area of roughly 100 000 km², showing that the mead, *iQhilika*(Xhosa) *Kari* (Khoi-San/Afrikaans), is produced using a very similar process throughout the region. This involves the roots of a *Trichodiadema* sp. plant (*imoela* – Xhosa, *karimoer* – Afrikaans/Khoi-San), honey, extract of brood and/or pollen and water. Various other fruit sugar sources were also found to be added at times producing seasonal beverages with unique organoleptic properties.

A model traditional *iQhilika* production operation was investigated in order to describe the main features of the process. Biomass immobilised on *Trichodiadema* root segments was found to be distributed evenly through the profile of the bioreactor resulting in a well mixed fermentation and a productivity of

0.74 g EtOH/l/h. In the initial stages of fermentation, the ethanol yield was highest in the mid-regions of the bioreactor, but with time the regions closer to the surface, which had atmospheric contact had a higher yield. This phenomenon was attributed to aerobic fatty acid synthesis which allowed the yeast close to the surface to function more efficiently despite rising ethanol concentrations. The mead contained 44.25 g/l (7 % volume) ethanol produced in a fermentation time of 43.5 h.

Yeast biomass in the traditional process was either immobilised in the form of flocs or attached to the *Trichodiadema intonsum* support. Electron microscopy revealed that the cells were covered in a layer of extra-cellular polymeric substance apparently assisting the immobilization, and which was populated by a consortium of yeasts and bacteria.

Yeasts isolated from *iQhilika* brewed in two regions separated by 350 km were found to be very closely related *Saccharomyces cerevisiae* strains as determined by molecular genetic analysis. The traditional beverage was found to contain populations of Lactic acid bacteria (LAB), which are known spoilage organisms in other beverages. Spoilage characteristics of these organisms matched descriptions of spoilage provided by the IKS survey. Other possibly beneficial LAB, which may contribute useful flavour compounds, were also found to be present in the system.

The basic functional aspects of the traditional process were used to design a continuous bench-scale tower bioreactor and process development was based on the IKS survey. This consisted of a packed bed bioreactor, consisting of 2 mm³ *T. intonsum* root segments, immobilising a novel

Saccharomyces cerevisiae strain isolated from a traditional batch of *iQhilika*.

The bioreactor performed well with a yield of close to the theoretical maximum and an ethanol productivity of 3.45 g EtOH/l/h.

The parameters of the 5.6 l/d bench-scale bioreactor were used to design a full-scale production bioreactor with a planned maximum output of 330 l/d.

This bioreactor had a productivity of 0.19 g EtOH/l/h. The organoleptic properties of the product produced were considered by a taste panel to be better than those of the product of the bench-scale tower bioreactor.

This research was based on the development of IKS which imposed a number of constraints and obligations on the project to ensure environmental, and social, in addition to financial viability of the scale-up operation.

Makana Meadery was established in partnership with Rhodes University as an empowerment company which, in addition to undertaking the commercialisation of the *iQhilika* process, would also develop methods for the production of scarce ingredients traditionally unsustainably sourced from fragile ecosystems, provide beekeeping training and the manufacture of beehives.

These developments are reported and the final commercialised *iQhilika* mead produced as a result of this research was selected to be served as the welcoming drink at the inauguration of Mr Thabo Mbeki as the President of South Africa.

Table of contents

Title page	i
Abstract.....	iii
Table of contents	vi
List of figures.....	xiii
List of tables.....	xvi
Acknowledgements.....	xviii
Glossary and abbreviations	xxi

Chapter 1 *iQhilika* – an African Mead

1.1 Introduction	1
1.1.1 Traditional mead making in Africa	5
1.1.2 The importance of mead in the informal African economy.....	9
1.1.3 Traditional South African mead making as a suppressed indigenous knowledge system	10
1.1.4 Khoi-San mead making	11
1.1.5 <i>Kari/iQhilika</i> in the literature.....	13
1.1.6 Other African meads.....	15
1.1.7 The Eastern Cape as a multi-cultural refuge of Khoi-San mead.....	18
1.1.8 Commercialisation of mead starting with <i>iQhilika</i>	19
1.2 Research hypothesis	20
1.3 Research objectives	21

Chapter 2 - The traditional *iQhilika* production system

2.1 Introduction	22
2.2 A survey of <i>iQhilika</i> production in the Eastern Cape	22
2.2.1 The Oral <i>iQhilika</i> tradition	24
2.2.2 The origin of <i>iQhilika</i> according to oral history	29

2.2.3 Economic significance of <i>iQhilika</i>	31
2.2.4 Cultural significance of <i>iQhilika</i>	33
2.3 Analysis of a model <i>iQhilika</i> production system	34
2.3.1 Methods	34
2.3.1.1 Selecting a model traditional bioreactor to analyse.....	34
2.3.1.2 Sampling	35
2.3.1.3 Alcohol and sugar analysis.....	36
2.3.1.4 Electron microscopy	37
2.4 Results	37
2.4.1 Analysis of a model <i>iQhilika</i> bioreactor	37
2.4.1.1 Bioreactor vessel	37
2.4.2.2 Biomass	38
2.4.2.3 Substrate preparation	38
2.4.2.4 Biomass analysis	39
2.4.2.5 Fermentation profiles.....	41
2.4.2.6 Biomass and root analysis.....	45
2.5 Discussion.....	47
2.5.1 Biomass immobilisation	47
2.5.2 Bioreactor operation	51
2.5.3 Bioreactor classification	53
2.6 Conclusion	54

Chapter 3 - Characterisation of the dominant *iQhilika* mycoflora

3.1 Introduction	56
3.1.1 Spontaneous fermentations.....	56
3.1.2 The ecology of spontaneous wine fermentations	56
3.1.3 Inoculated fermentations	57
3.1.4 The evolution of fermented beverages	57
3.1.5 Potential sources of microbes in <i>iQhilika</i>	58
3.1.5.1 Honey.....	58
3.1.5.2 Plant roots.....	59
3.2 Objectives	59

3.3 Materials and methods	59
3.3.1 Sample sites	60
3.3.2 Yeast Isolation	61
3.3.3 Identification of yeast isolates.....	61
3.3.3.1 Phenotypic identification.....	61
3.3.3.2 Physiological identification techniques	61
3.3.3.3 Molecular identification	62
3.3.4 Fermentation characteristics	63
3.3.5 Enzyme profiles of isolates.....	65
3.3.6 Electron microscopy	65
3.4 Results	66
3.4.1 Phenotypic identification.....	66
3.4.2 Physiological identification.....	66
3.4.3 Molecular identification	68
3.4.4 Yeast enzymes	70
3.4.5 Electron microscopy	71
3.4.6 Organoleptic analysis by traditional brewers.....	74
3.4.7 Fermentation characteristics	74
3.5 Discussion.....	76
3.6 Conclusion	79

Chapter 4 - Lactic acid bacterial populations in traditional *iQhilika*

4.1 Introduction	81
4.1.1 LAB in African traditional beverages other than mead.....	82
4.1.1.1 Indigenous beers in Africa	83
Finger millet beverages and millet beverages.....	83
Sorghum beverages	84
Maize beverages.....	84
4.1.1.2 LAB in the indigenous wines of Africa	85
4.1.2 Lactic acid bacterial fermentations	85
4.1.3 Lactic acid bacteria identification.....	86
4.2 Objectives	87

4.3 Materials and methods	87
4.3.1 Samples	87
4.3.2 LAB isolation	87
4.3.3 Electron microscopy	88
4.4 Results	89
4.4.1 Isolation.....	89
4.4.2 Carbohydrate assimilation/utilization profiles	89
4.4.3 Electron microscopy	90
4.5 Discussion.....	91
4.6 Conclusion	93

Chapter 5 - Development of a bench -scale *iQhilika* production system

5.1 Introduction	94
5.1.1 Immobilised biomass, glycerol production and flavour.....	95
5.1.2 Honey storage	96
5.1.3 Continuous fermentation.....	97
5.1.3.1 Continuous fermentation of beer	97
5.1.3.2 Molasses and corn steep liquor.....	99
5.1.3.3 Cider	100
5.1.3.4 Wine production.....	101
5.1.3.5 Other continuous fermentation systems.....	102
5.1.3.6 Potential for the continuous fermentation of <i>iQhilika</i>	103
5.2 Objectives	103
5.3 Materials and methods	103
5.3.1 Bioreactor design	103
5.3.2 Support configuration.....	104
5.3.3 Inoculum and support preparation.....	105
5.3.4 Yeast immobilisation and growth.....	105
5.3.5 Electron microscopy of flocs.....	106
5.3.6 Bioreactor stocking	107
5.3.7 Bioreactor biomass development cycle	107
5.3.8 Bioreactor operation	107

5.3.9 Sample analysis.....	110
5.4 Results	111
5.4.1 Support density	111
5.4.2 Support configuration.....	111
5.4.3 Flocculation characteristics of strain F	113
5.4.4 Electron microscopy of flocs.....	114
5.4.5 Bioreactor operation	115
5.4.6 Bioreactor fermentation profile	118
5.4.7 Packed bed characterisation	120
5.4.8 Organoleptic and alcohol analysis of aged samples	122
5.5 Discussion.....	122
5.6 Conclusion	127

Chapter 6 - Development of a commercial-scale *iQhilika* tower bioreactor

6.1 Introduction	129
6.1.1 Honey supply	131
6.1.2 Availability of root supports.....	131
6.1.3 Scale up strategies	132
6.2 Objectives	134
6.3 Materials and methods	134
6.3.1 Determination of the size of the bioreactor.....	134
6.3.2 Root support preparation.....	134
6.3.3 Selection of scale up criteria.....	134
6.3.3.1 Flow rate	135
6.3.3.2 Gas evolution	136
6.3.3.3 Time	137
6.3.4 Stainless steel tower bioreactor construction.....	138
6.3.5 Bioreactor operation	138
6.3.5.1 Sterilization	138
6.3.5.2 Inoculation.....	138
6.3.5.3 Media and media sterilization	139
6.3.5.4 Biomass development	139

6.3.5.5 Bioreactor operation	140
6.4 Results	140
6.4.1 Assessing the effectiveness of formula 6.1	140
6.4.2 Scale up construction	143
6.4.3 Stainless steel tower bioreactor operation	143
6.4.4 Validation of predicted bioreactor characteristics.....	143
6.4.5 Full -scale tower bioreactor productivity	144
6.4.6 Organoleptic analysis	147
6.5 Discussion.....	147
6.6 Conclusion	147

Chapter 7 - Issues affecting the development of *iQhilika* production

7.1 General Introduction	149
7.2 Cultivation of <i>Trichodiadema intonsum</i> for root support provision	149
7.2.1 Introduction	151
7.2.2 Objectives	151
7.2.3 Materials and Methods	151
7.2.3.1 <i>Trichodiadema intonsum</i> cultivation	151
Obtaining sample plants	151
Obtaining Seeds	152
Vegetative cuttings.....	152
7.2.3.2 Assessment of the effectiveness of cultivation.....	152
7.2.4 Results	153
7.2.4.1 Seedlings and vegetative cuttings.....	153
7.2.4.2 Growth Rates of <i>Trichodiadema intonsum</i> seedlings.....	153
7.2.4.3 Acceptability of cultivated roots to traditional brewers	156
7.2.4.4 Pests and problems	156
7.2.5 Discussion.....	156
7.2.6 Conclusion	157
7.3 Provision of pollen for <i>iQhilika</i> making.....	157
7.3.1 Introduction	157
7.3.2 Materials and methods	159

7.3.2.1 Pollen trapping.....	159
7.3.3 Results	159
7.3.3.1 Pollen trapping.....	159
7.3.3.2 Pollen testing in <i>iQhilika</i> production.....	160
7.3.4 Conclusion	161
7.4 Commercialisation of <i>iQhilika</i> as a rural economic enterprise	161
7.4.1 Introduction	161
7.4.2 Objectives	162
7.4.3 Materials and methods	162
7.4.3.1 Factory Design.....	162
7.4.3.2 Beekeeping support products.....	162
7.4.3.3 Beekeeping	163
7.4.4 Results	163
7.4.3.1 Factory Design.....	163
7.4.3.2 Beekeeping support products.....	164
7.4.3.3 Beekeeping	165
7.4.4 Discussion.....	167
7.4.5 Conclusion	167
7.5 Concluding Remarks	167
7.6 Future Work	168
9 References.....	172

List of figures

Figure 1. 1 Global mead production based on	2
Figure 1. 2 The compiled price data (England) for mead	3
Figure 1. 3 A 40 year old bottle of mead from	4
Figure 1. 4 A range of meads from around the world.....	4
Figure 1. 5 To place the African mead industry.....	9
Figure 1. 6 A cave painting from the Salem area.	12
Figure 1. 7 A map compiled from linguistic.....	18
Figure 2. 1 The region in which <i>iQhilika</i> making.....	23
Figure 2. 2 <i>Trichodiadema stellatum</i> (A) and.	24
Figure 2. 3 Beekeepers, Mr Vuyani Ntantiso (right)	25
Figure 2. 4 The prickly pear (<i>Opuntia ficus-indica</i>).....	26
Figure 2. 5 The jacket plum, <i>Pappea capensis</i>	26
Figure 2. 6 Kei-apple (<i>Dovyalis caffra</i>) fruits seen.	27
Figure 2. 7 A pineapple vendor on the streets of	27
Figure 2. 8 A Xhosa women picks 'umgwenya'	28
Figure 2. 9 The large juicy fruits of <i>Carissa macrocarpa</i>	29
Figure 2. 10 The Karee tree (<i>Rhus lancea</i>)	31
Figure 2. 11 Ms Zintombi Zabo, an <i>iQhilika</i> merchant.....	32
Figure 2. 12 The death's head moth, so called	34
Figure 2. 13 The traditional bioreactor vessel of Mr Makwana.	35
Figure 2. 14 The dry biomass preparation.....	38
Figure 2. 15 The rough distribution of the <i>T.intonsum</i> supports.....	39
Figure 2. 16 The dry weight of freely suspended cells.....	40
Figure 2. 17 Dry weight immobilised biomass profile	41
Figure 2. 18 The bioreactor after 2 hours of fermentation.....	41
Figure 2. 19 Bioreactor sugar and ethanol concentrations	42
Figure 2. 20 The yield of the bioreactor over time	42
Figure 2. 21 The profile of the bioreactor after 6.5 hours.....	43

Figure 2. 22 The profile of the bioreactor in terms of Ethanol	43
Figure 2. 23 The profile of the bioreactor after 31 hours.	44
Figure 2. 24 The ethanol yield profile of the bioreactor after 31 hours.	44
Figure 2. 25 Time profile of pH in the bioreactor.	45
Figure 2. 26 SEM of the surface of a piece of T.intonsum	45
Figure 2. 27 An SEM showing the inside of a cavity	46
Figure 2. 28 An SEM of a floc which formed spontaneously.....	46
Figure 2. 29 The biomass preparation after draining of the bioreactor	47
Figure 3. 1 A map of South Africa showing sample sites	60
Figure 3. 2 Comparison of the partial sequences.....	68
Figure 3. 3 A consensus tree.....	69
Figure 3. 4 A neighbour joining tree	70
Figure 3. 5 A scanning electron micrograph (SEM).	71
Figure 3. 6 An SEM of a typical example of Strain F.....	72
Figure 3. 7 An SEM of an older yeast cell of strain F	72
Figure 3. 8 On nutrient limiting media containing honey.	73
Figure 3. 9 The central cell in this SEM appears to have lysed.	73
Figure 3. 10 The growth and flocculation of the various strains.....	75
Figure 4. 1 A scanning electron micrograph (SEM)	90
Figure 4. 2 This SEM shows a floc of yeast and bacteria..	90
Figure 5. 1 The tower bioreactor used by Royston (1966).....	98
Figure 5. 2 The continuous tower bioreactor of Chen et al. (1994).....	100
Figure 5. 3 The experimental set-up used by Nedovic et al. (2000)	101
Figure 5. 4 The glass tower bioreactor	104
Figure 5. 5 The glass tower bioreactor was operated at	108
Figure 5. 6 Settling velocities obtained for 2mm	111
Figure 5. 7 Settling velocities obtained for 5mm	112
Figure 5. 8 Settling velocities obtained for 10mm	112
Figure 5. 9 The Trichodiadema intonsum plant.....	113
Figure 5. 10 Growth of strain F on glucose.	113
Figure 5. 11 Growth of Strain F on fructose	114

Figure 5. 12 Honey growth curves for strain F	114
Figure 5. 13 A clump of flocculated strain F cells.....	115
Figure 5. 14 The glass tower bioreactor in operation.	115
Figure 5. 15 The packed bed region of the glass tower bioreactor	116
Figure 5. 16 The transitional zone.	116
Figure 5. 17 Operation parameters.	117
Figure 5. 18 Operation parameters	117
Figure 5. 19 Operation parameters	118
Figure 5. 20 The depth profile of the tower glass bioreactor	119
Figure 5. 21 Data comparing the yeast bed (□).....	119
Figure 5. 22 This figure shows the ethanol yield	120
Figure 5. 23 Bioreactor bed productivity over time.....	122
Figure 6. 1 The tubular heat exchanger design.....	139
Figure 6. 2 The dimensions of the stainless steel tower bioreactor	142
Figure 6. 3 The grade 304 stainless steel tower bioreactor	143
Figure 6. 4 Operation parameters	144
Figure 6. 5 Productivity for the active bed region	145
Figure 6. 6 Productivity of the packed bed region	146
Figure 7. 1 This graph shows the volumetric displacement	153
Figure 7. 2 The reasonably uniform growth of plants at 6	154
Figure 7. 3 After a year of growth	154
Figure 7. 4 Where branches of <i>T.intonsum</i> contact.	155
Figure 7. 5 A patch of <i>Trichodiadema intonsum</i> in.....	155
Figure 7. 6 Pollen yields.	159
Figure 7. 7 Pollen yields	160
Figure 7. 8 Bottling Makana Meadery's first	164
Figure 7. 9 The sawmill converted low quality logs	164
Figure 7. 10 The manufacture of beehives.....	165
Figure 7. 11 Beekeeping exchanges to Canada	166
Figure 7. 12 A group of trainee beekeepers	166

List of tables

Table 3. 1 API 32C carbon assimilation	67
Table 3. 2 API Zym Enzyme profiles	71
Table 3. 3 Outcomes of organoleptic assessments	74
Table 3. 4 The fermentation characteristics of the isolates.....	74
Table 4. 1 Tentative identifications of isolates.....	89
Table 4. 2 The frequency of the different LAB.....	91
Table 5. 1 An analysis of Jamieson Reservoir water	108
Table 5. 2 Ethanol productivity.	121
Table 6. 1 Values used to solve formula 6.1	140
Table 6. 2 Values used to solve formula 6.1	141
Table 6. 3 Ethanol productivity calculated for.....	145
Table 6. 4 A comparison of the productivity	146

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Glossary and abbreviations

This thesis has a multidisciplinary nature. To avoid confusion the following terms and abbreviations are defined.

Adjunct – this word is employed to describe the addition of ‘an adjunct’ to a fermentation mixture to achieve an effect.

Bioreactor – the containment vessel of any biotechnology based production process (Smith 2004).

Brewer – traditional mead-makers are referred to as brewers, although this is not technically correct as brewers make beer. The term meader or mazer is however not generally known.

Brood – the wax combs in a beehive containing larval bees.

EtOH - ethanol

IKS – Indigenous Knowledge System. Sociological jargon for local traditional knowledge. The term has become prevalent in South Africa due to its adoption by the National Research Foundation.

LAB – Lactic acid bacteria

Must – In some cases used to describe a honey and water solution.

Specific gravity – This term, frequently used in wine making circles refers to the relative density of a solution to pure water. Generally it is measured with a hydrometer and provides a rough quantification of solutes, such as sugars, present.

W4W – Working for water programme of South African government. Involves clearing invasive plants to ensure water security.

Chapter 1 *iQhilika* – an African Mead

"In the last 30 years, the torch of sustainable development has travelled from Europe, to the Americas, through Asia, and now burns in Africa. After a protracted journey, it has arrived in the continent that is the cradle of humanity."

South African President Mr Thabo Mbeki at the World Summit on Sustainable Development 2002.

1.1 Introduction

Fermented beverages have played an important part in the evolution of most human cultures (Dietler 1990) and mead, produced by the fermentation of honey (Gayre 1948, Steinkraus & Morse 1966, Crane 1999) may be among the most ancient of these beverages. Mead can be produced by natural factors such as the flooding of a beehive and in this sense mead would certainly predate human existence (Acton & Duncan 1984). Among the other ancient beverages, wine is produced through the fermentation of fruits (Thudichum & Dupre 1872, Crane 1999), while the fermentation of sugar extracted from grain via the malting process yields beer (Hough 1984, Crane 1999). Although also ancient, these beverages are probably largely anthropogenic in origin.

In small societies alcoholic beverages are generally produced and consumed at a restricted local level, but as societies become more complex, trade in beverages occurs (Dietler 1990). This necessitates improvements in the quality of the beverages produced to increase shelf life and consumer appeal, which in turn increases profits and leads to greater production and a larger area over which the products can be marketed.

The evolution of the wine and beer industry has occurred as a result of the scientific elucidation of the traditional production systems associated with these beverages, allowing the beverages to make the transition from traditional to commercial production systems producing beverages of a consistently marketable standard (Stewart & Russell 1998, Pretorius 2000). The research and development process for the wine (Pretorius 2000) and beer industry (Atkinson 1991, Stewart & Russell 1998) is ongoing with the

wine industry producing approximately 27 billion litres per annum (Pretorius & Bauer 2002) and the beer industry 142 billion litres in 2001 (World Advertising Research Centre 2004). The scientific and popular literature for wine and beer is substantial with entire specialised libraries devoted to each beverage, such as the Napa Valley Wine Library Association in St. Helena, California and the National Brewing Library at Oxford Brookes University.

Similar data is not readily available for mead. An on-line investigation of 12 of 23 commercial meaderies revealed that these producers had combined sales of 205 000 litres per annum and an installed capacity of 1 023 650 litres. Davydova (2004) reports that a revival in mead production has occurred in Russia with sales in the region of 2 022 000 litres per annum. Figure 1.1 shows the global mead production determined in this survey. No data was obtained for countries such as France and Poland, and data for the UK did not distinguish between cider, perry and mead, hence the production in these countries is likely to be higher as only definite data was included in the survey. It is also important to note that famous 'meads' such as those of Lindesfarne monastery are not fermented products, but fortified beverages containing grape juice and spirits.

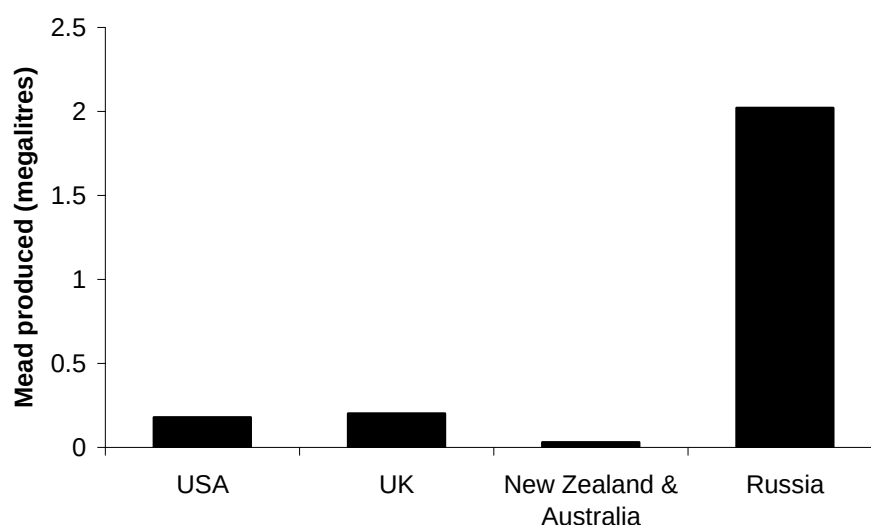


Figure 1. 1 Global mead production based on figures obtained by questioning meaderies and a published report (Davydova 2004).

Mead was at one point one of the most commonly consumed beverages in much of Europe, Russia and India (Gayre 1948). Mead was also commonly made in China and Tibet with its consumption performing both cultural and medicinal functions (Yaochun 1984). Various factors caused the decline of mead, with the main factor being price, due largely to increasing scarcity in honey caused by beekeeping technology not advancing rapidly enough to match honey supply with population growth (Gayre 1948). As a consequence, mead was not able to compete with wine on price (Figure 1.2).

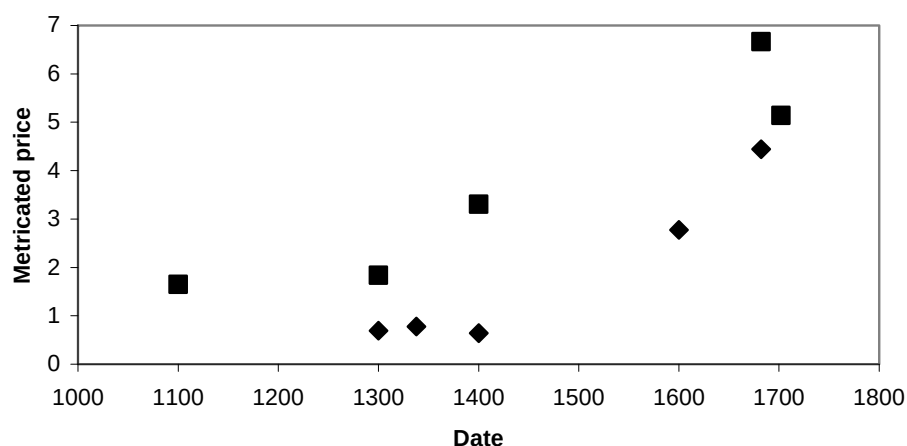


Figure 1. 2 The compiled price data (England) for mead (■) and premium wine (♦) (Gayre 1948, Blackall *et al.* 1984). Mead was generally a more expensive beverage than even the best wines.

A bibliography of honey wines was compiled by R.A. Morse in 1972 (www.gotmead.com/mead-research/kime.shtml 10/2/2005) and listed 159 references. It is evident that the mead literature is proportionately much smaller than either the wine or beer literature.

Mead fermentations observed by Steinkraus & Morse (1966) were generally very slow, hence they developed a nutrient and vitamin solution which could be added to new mead batches to encourage fermentation. This increased the rate of fermentation of mead allowing it to be produced in weeks rather than months. This in essence represented the first major scientific innovation in mead making in 5000 years (www.gotmead.com/mead-research/kime.shtml

10/2/2004). Kime *et al.* (1991 a & b) advanced the science of mead making by investigating ways of improved preparation of mead fermentation media (must), prior to fermentation, enhancing the shelf life and bouquet of the mead produced. Karuwanna *et al.* (1993) experimented with meads produced with small quantities of grape juice included to enhance fermentation and bouquet.



Figure 1. 3 A 40 year old bottle of mead from the London Winery, Ontario Canada. The sediment observed to commonly form in mead by Kime *et al.* (1991 a & b) is evident in this bottle.

The composition of commercial mead varies as much as its packaging (Figure 1.3 & Figure 1.4), with commercial meads ranging in alcohol volume from 6.4% -16.6% , pH from 3.45 – 4.00, with residual sugar ranging from 7.1 g/l to 370.5 g/l (Steinkraus & Morse 1973). Mead produced in Thailand fell within the same range (Karuwanna *et al.* 1993).



Figure 1. 4 A range of meads from around the world.

It is apparent that due to a lack of active research into mead production, and the largely non-peer reviewed nature of mead knowledge generation, that the majority of information transfer would fall into the category of *word of mouth* advice between one mead maker and another. When mead was commonly made in Europe in the Dark Ages and Middle Ages (Gayre 1948), scholars did not possess the scientific or biotechnological insights to elucidate the traditional mead making systems. This is highlighted by the following record which is reproduced in Crane (1999) from a far more ancient work:

“...hydromel was prepared in the usual way, but in the high Vosges to the south-east the whole contents of a skep of bees was crushed and the blackish mixture added to water to make *miessaule*...” Crane (1999) p. 516

The fact that an entire skep, a primitive straw beehive (Root *et al.* 1975) which would have included pollen and brood, was added to the mead mixture, suggests an underlying traditional understanding of means of eliminating the nutrient deficiencies in pure honey which cause the slow fermentation of mead. The addition of pollen to the honey would perform the same function as the nutrient and vitamin solutions of Steinkraus & Morse (1966). In this way, traditional mead makers would have enhanced their fermentations and produced mead more rapidly. Hence the scientific addition of nutrients to speed up mead fermentation is probably a modification of a traditional mead making strategy that was lost to European mead makers hundreds of years ago before the development of a scientific community which could record it accurately.

1.1.1 Traditional mead making in Africa

Mead making in Africa is what may be called an ‘invisible industry’. In South Africa under British rule in the early 1900s, traditional mead making was outlawed (Juritz 1906). Various liquor laws were passed during the last century culminating in the South African Liquor Products Act 27 of 1989, which governs the production of alcohol in South Africa. Under the regulations

to the Act published on the 6 March 1998 the following general requirements are listed for mead which must:

- a) be produced by the alcoholic fermentation by means of wine yeast of a mixture of honey and water;
- b) have an alcohol content of at least 4% and not more than 16.5%;
- c) have a sulphur dioxide content of not more than 200 milligrams per litre, of which, not more than 50 milligrams per litre may be in the form of free sulphur dioxide;
- d) have a volatile acid content which calculated as acetic acid does not exceed 1.2 grams per litre; and
- e) have a flavour, taste and character which clearly distinguishes it from that of any other liquor product or class of liquor products.

This legislation excludes the majority of traditional mead makers from being active players in the legal formal alcohol industry. Similar suppression of traditional knowledge occurred throughout colonial Africa. Hence the traditional mead industry in Africa has tended to operate largely outside the confines of the formal economy, frequently in countries with little research infrastructure. Production has been hidden behind layers of cultural suspicion towards outside observers, and the few colonial observers who have been privy to mead making information, frequently overlooked the significance of these beverages due to a culturally constrained outlook and a lack of microbiological training. An example of this is found in three reports on beekeeping in Tanzania (Ntenga & Mugongo 1991), Zambia (Forestry Department Report 1992) and Botswana (Clauss 1983). Of these three publications, only the one written for Tanzania, by Tanzanians actually provides names and detailed descriptions for the types of mead made in Tanzania. This publication also highlights the cultural and economic significance of these beverages and suggests mead making as a wealth generating activity. The other two publications view the making of mead as a nuisance which detracts from the business of producing and exporting honey to earn foreign exchange.

This bias among colonial observers is neatly summed up by Nelson Mandela, *Long walk to freedom* (1995) p.16, “The education I received was a British education, in which British ideas, British culture and British institutions were automatically assumed to be superior. There was no such thing as African culture.”

The majority of literature available on mead in Africa does not categorize the African honey beverages correctly, and African mead is generally called honey beer (e.g. Juritz 1906, Gayre 1948, Crane 1999). This is despite the fact that the majority of recipes listed for mead in Africa do not involve grain. It appears that this is again due to a differential in respect towards the ancient mead makers of Europe and the ‘primitive’ (Gayre 1948) mead makers of Africa. As will be shown here, African mead making is a biotechnologically sophisticated process and the use of the word ‘primitive’ in conjunction with the African mead industry therefore suggests negative observer bias in the literature.

In some cases, these historical observations were more accurate and not as disdainful in tone. Lawrence Green, *In the land of afternoon* (1949) p.161 wrote, “Much wild honey is gathered because honey means honey beer. Coloured people in the Picketberg Sandveld still make it every year, and they prefer honey cakes containing young bees. That, they say, starts the fermentation properly. They use *kareemoer*, a powdered root, as yeast; and when water is added there is a strong drink such as primitive people made in many lands when the world was young.”

In other parts of Africa, mead production has been noted by foreign government agencies. Ethiopia produces 24 000 metric tons of honey per annum, of which 80% is converted to *tej*, (US Embassy 1997). *Tej* and *mies* are the names for mead in Ethiopia (Vetter 1997, Tadesse *et al.* 2005). Ethiopia has 10 million managed beehives and has the most intensive beekeeping activity in Africa. The *tej* and *mies* produced in Ethiopia can be estimated to be in the region of 173 million litres per annum.

The significance of bees to Ethiopians is demonstrated by this excerpt from their national anthem:

*“...Ethiopia land of our fathers
the land where our God wants to be
like bees to a hive swiftly gather...”*

N.Adgaba pers.com.

In Zambia, a beekeeping study for the North Western Province (Forestry Department Report 1992) indicated that the province had an export production of 53 metric tons of wax per annum between 1934 and 1964. Most of the honey produced from managed beehives and wild colonies was used to make ‘honey beer’ with the by-product of this being beeswax (Forestry Department Report 1992). Approximately 90% of this beeswax was discarded (Forestry Department Report 1992), hence the annual wax production was probably close to 530 metric tons. Given that beeswax can support 30 times its weight in honey (Root 1975), and that the Zambian mead is made by diluting 1 kilogram of honey to 7 litres of water (Forestry Department Report 1992), this gives a mead production of around 111 million litres (Figure 1.4). This does not include the possibility of extension of the honey with other sugar sources such as fruit juice.

The estimated annual beeswax production of Africa is 8000 metric tons (Hepburn & Radloff 1998). Assuming the ratio of 30 parts honey per part beeswax (Root *et al.* 1975), this translates to an annual crop of 240 kilotons of honey. As has been discussed earlier the majority of the honey produced in Zambia and Ethiopia is used for mead making. The same is true for Tanzania (Ntenga & Mugongo 1991) and a large percentage of wild hives in Botswana are raided for mead production (Clauss 1983). If the remainder of Africa follows a similar trend, the majority of the total 240 kiloton annual African honey crop is possibly used for mead making, which if correct, would give projected annual mead production in the region of 1 to 1.7 billion litres (Figure 1.5).

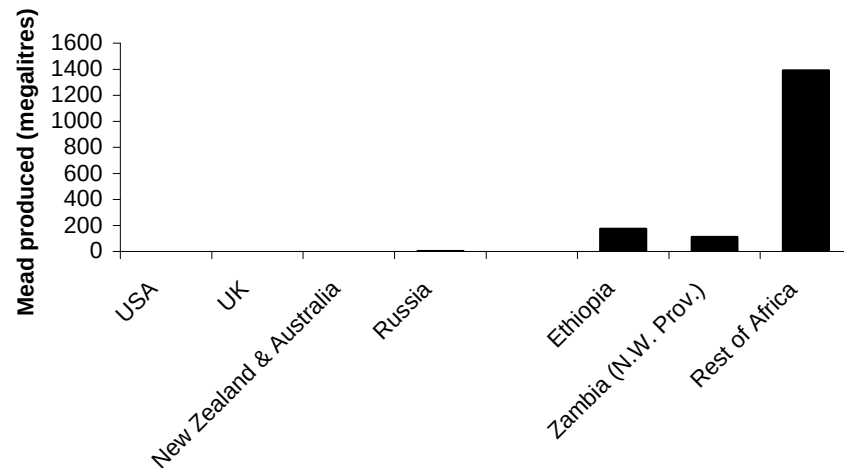


Figure 1. 5 To place the African mead industry in perspective, it is useful to include the data from Africa with the data from Figure (1.1). This clearly shows that mead is an important beverage in Africa and has not become as rare as it has in other parts of the world.

This suggests that mead making in Africa is a large traditional industry, the size of which has not been quantified before. Clauss (1983), Ntenga & Mugongo (1991), and the Forestry Department Report (1992) all mention that mead is produced for local sale and barter. This suggests a large population of mead makers each make a small portion of the total African product. A large population of mead makers has the potential to support a sizeable indigenous knowledge system, with recipes being passed from generation to generation.

1.1.2 The importance of mead in the informal African economy

In Grahamstown the traditional Xhosa mead *iQhilika* is typically sold by the cup at a price of R 10 - R 15 per litre (discussed further in Chapter 2). As a comparison it is interesting to note that one Big Mac burger costs US\$ 1.86 in South Africa and an average of US\$ 2.95 in the US (The Economist, 2004). The price of a Big Mac burger is regarded as a useful measure of the purchasing power parity of a currency (Ong 1997).

In the North Western Province of Zambia mead is used as an item of barter and trade, whereby a farmer will exchange mead for the lease of oxen for

ploughing (Forestry Department Report 1992). In Tanzania the trade in mead and mead ingredients is a major part of the traditional economy (Ntenga & Mugongo 1991).

If one uses the South African *iQhilika* price as a standard for Africa, and the updated adjustment factor of Ong (1997) it gives an industry with an approximate value of US\$ 4.1 billion (million million) per annum.

As is evidenced by figures quoted so far, the majority of this value is probably traded in items other than money, hence this industry is largely invisible to the financial sector. It does however play an important part in ensuring sustainable livelihoods in a continent where this is desperately needed.

The fact that the informal African mead industry operates outside the formal economy means that it does not generate tax revenues, and as a result, this informal industry will not support much state sponsored research. It will struggle to remain competitive with beverage industries which do contribute tax revenues and which benefit from state research and development.

Hence without some form of state investment in formalising the African mead making industry, so that it can pay its dues, it will become uncompetitive with other beverages and eventually the traditional African mead industry will fail.

1.1.3 Traditional South African mead making as a suppressed indigenous knowledge system

In 1994 with the advent of democracy in South Africa, many aspects of society changed and a more open cultural system began to develop. Various laws which had restricted certain cultural activities were repealed. The South African National Research Foundation (NRF), formerly the Foundation for Research Development (FRD) designated Indigenous Knowledge Systems (IKS) as a key research focus area (www.nrf.ac.za/focusareas/iks 25/1/05). The IKS focus on indigenous knowledge (IK) is divided into themes namely;

“The production, transmission and utilisation of indigenous knowledge and technology ;

The role of IK in nation building ;

IK at the interface with other systems of knowledge. ;

Introducing IKS into the mainstream of education.”

(www.nrf.ac.za/focusareas/iks, 25/1/2005)

A primary objective of the IKS focus area is to research IKS which has been suppressed.

The South African government had, through the recognition of IKS, made the first step in providing an opportunity to research the African mead industry and possibly formalise aspects of it.

To begin this research, the African mead industry would need to be scrutinised to determine its origins.

1.1.4 Khoi-San mead making

The Khoi-San are one of the most ancient peoples on Earth (Comrie *et al.* 1996, Stoneking & Soodyall 1996). The making of mead by these people has been recorded in many places (Green 1949, Pahl *et al.* 1989, Silva *et al.* 1996, Crane 1999).

A small population of Khoi-San currently occupy the dry regions of Botswana, South Africa and Namibia, whereas 10 000 years ago the Khoi-San were predominant in much of Africa (Comrie *et al.* 1996). The rock art of the Khoi-San frequently depicts honey gathering (Figure 1.6), and a global register of rock paintings depicting bees has been established by Crane 1999.



Figure 1. 6 A cave painting from the Salem area, 15 km outside Grahamstown in South Africa. The picture shows a Khoi-San honey gatherer reaching up and removing honeycombs from a beehive on a cliff. The painting is believed to be 500-1000 years old. Photograph courtesy of Dr J. Binneman, Archaeology Department, Albany Museum, Grahamstown.

The Bantu peoples of Africa, who are now dominant throughout the original Khoi-San zone which covered roughly half of the length of Africa, are believed to have originated somewhere in West Africa (Comrie *et al.* 1996). Their movement southwards and eastwards resulted in frequent contact with the Khoi-San and intermarriage was not uncommon (Comrie *et al.* 1996). This in turn resulted in the culture of the Khoi-San being transferred to the peoples who largely displaced them.

The isiXhosa language of the Xhosa people, the most southern of the Bantu people, has incorporated many elements of the Khoi-San languages, such as clicks and many words (Comrie *et al.* 1996). Another group to contact the Khoi-San were the Afrikaans people who moved up from the Cape Colony in the 1700s (Comrie *et al.* 1996). The Khoi-San in South Africa hence have largely become either Xhosa speakers or Afrikaans speakers and much of their traditional knowledge has passed into these cultures and languages.

The Khoi-San word for mead is *karee/kari/karie* (Branford & Branford 1991, Silva *et al.* 1996) and the impact of the Khoi-San on Afrikaans is evident in the

following definition of mead from the Van Shaik English/Afrikaans Dictionary (Kritzinger et al.1981):

“Mead - heuningdrank, karie, mee.”

The impact of the Khoi-San on Xhosa mead making is evident in the following mead definitions:

“iQhilika n. beer made from honey, mead”

A New Concise Xhosa-English Dictionary, McLaren 1978

“iQhilika n. Kh. !Kharib, liquor brewed from honey. The art of making this beer was acquired from the Khoi....”

The Greater Dictionary of Xhosa, Pahl et al. 1989

The !Kharib were a Khoi tribe (Nienaber & Raper 1983).

1.1.5 Kari/iQhilika in the literature

Karee/kari is made by mixing honey, water and the roots of plants of the genera *Trichodiadema*, *Euphorbia* and *Anacampsestros*, known as *karriemoer* which are added to provide yeast (Silva et al. 1996).

Juritz (1906 p. 40) noted that ‘*Eqilika*’ produced by peoples within the present day Eastern Cape region of South Africa was an intoxicating beverage and that “...this beer is very potent, and one teacupful is sufficient to capsize the strongest.” Juritz (1906) p.41, quotes a certain Mr Verschuur, who was the assistant resident magistrate in the New Brighton region of Port Elizabeth, as saying that, “...Qilika, or honey beer is prepared from the following ingredients: 2 lbs. of honey or golden syrup and a small quantity of crushed ‘Moola ye Qilika.’ The honey or syrup is mixed with hot water until thoroughly diluted; the Moola is then added to the receptacle-a (*sic*) large paraffin tin-filled with water. As soon as fermentation commences it is fit for use.” Another observer is quoted as noting that *iQhilika* is consumed 12 hours after mixing. The *iQhilika* was sold for sixpence a pint and represented a livelihood for the makers (Juritz 1906).

Laidler (1928) stated that the *kareemoer* plant, *Mesembryanthemum stellatum*, is one of the beer making roots. This plant is also noted by Juritz (1906) to be called *Kiri moer* or *Moola*. Smith *et al.* (1950) mentioned that *karee* is the Hottentot¹ word for honey beer. The same author mentions that the *kareemoer* plant is in some cases *Trichodiadema stellatum*. In all these references it is evident that a plant root adjunct is used to perform some function related to the initiation of fermentation.

Crane (1999) lists the following San honey prayer from the Kalahari region:

*"I am weak from thirst and hunger.
Abo Itse (Great Father), let me live....
Let me find sweet roots and honey,
Let me come upon a pool.
Let me eat and drink. Ho Itse (Great Father),
Give me what I must have"*

Although this prayer may deal merely with honey to eat, it is equally possible that the song deals with finding the ingredients of *kari/iQhilika*-mead making, as roots, honey and water are the ingredients.

The Khoi-San are an ancient people (Stoneking & Soodyall 1996), who at one point ranged over a large section of Africa (Comrie *et al.* 1996). Their honey gathering is recorded in rock art (Crane 1999). Their mead making has been noted by many to include the use of plant adjuncts to initiate fermentation (e.g. Green 1949, Silva *et al.* 1996, Crane 1999). It can be tentatively hypothesised that the mead making systems of the Khoi-San may have influenced the mead recipes of the peoples who today live where the Khoi-San used to. To evaluate this hypothesis, it is useful to review the reports of mead making in the rest of Africa.

¹ Hottentot – a member of the Khoi-San group of indigenous peoples (Branford & Branford 1991). It is now more politically acceptable to use the term Khoi-San.

1.1.6 Other African meads

Mead can be made by nature, without the intervention of man (Acton & Duncan 1984). This is confirmed by oral reports from a soldier, Mr J van Vreden, in the South African Defence Force (SADF) who took part in the invasion of the southern regions of Angola in the early 1980s. He detailed that during the build up to the spring rains hundreds of bee swarms would move through the baobab savannah. The upper region of an older baobab is frequently hollow, and swarms would settle here. The spring rains would come and the hollow would fill with water, driving the bees from the honeycombs as they became submerged. If the correct amount of rain fell, the honey would mix with the rainwater and turn to mead as the hive rotted. The San hunters who served in the SADF as trackers took great pleasure in finding such mead caches. The finding of such natural mead is likely to have inspired many a honey gatherer in ancient times to duplicate the effects that created the mead in the tree. With time and trial and error the sophistication of this mead making process would have advanced.

In producing the Ethiopian mead *tej*, discussed earlier in this chapter, the herb *geisho* (*Rhamnus prinoides*) is added as a part of the recipe (Vetter 1997). *Geisho* is cultivated as a commercial crop largely for use in flavouring *tej* (Vetter 1997). The plant, which contributes flavour to the beverage, is not added to initiate fermentation and after use is fed to donkeys (S. Vetter pers.com.). Hence, although the *geisho* is an adjunct, it is not added to initiate fermentation.

In Kenya mead was made by mixing water, honeycombs (with brood and pollen) and segments of loofa fruits (Crane 1999). Very importantly it is mentioned that the loofa fruits were dried between batches and probably acted as a source of yeast inoculation (Crane 1999). This is particularly relevant as Kenya is close to the northern-most range of the Khoi-San 10 000 years ago (Figure 1.6) and, as will be discussed later, the mode of conserving biomass in *KariliQhilika* fermentation involves drying fibrous root particles.

In Tanzania honey is sold in the comb, or containing comb to show that it has not been diluted. Local mead brewing customs require that the beeswax is not removed from the beverage (Ntenga & Mugongo 1991), hence there is little way of gauging the production of mead in the area as very little wax is sold. It is reported by these authors that mead is central to the culture in areas of Tanzania, with its consumption being required at every ceremony. Pollen and brood are reported to be added to the mead to increase its strength and to invigorate the yeast. Ancestral brewing gourds are handed from brewer to son and are, according to legend, imbued with the wisdom of the ancestral brewers who used them before. Special brewing gourds (*geskweg*) with volumes of up to 40 l, and liquid volumes of 20 l are reported. The volumes of honey produced in Tanzania are likely to far exceed those of the North Western Province of Zambia as the honey yield per hive is higher for Tanzania than Zambia (Ntenga & Mugongo 1991). It is therefore likely that the volume of mead produced in Tanzania is also sizeable.

The Tanzanian mead, *gesuda*, is a beverage of the Barbaig tribe who believe it is a knowledge gift given to twins by a God – the twins are reported to have been able to speak at birth and pass this knowledge on (Ntenga & Mugongo 1991). The Iraqw tribe of Tanzania is reported to make two types of mead, namely *ganguli* and *xangay*, with *xangay* being a modification of local beer by the addition of honey (Ntenga & Mugongo 1991).

The mead of Botswana is not named in the report of Clauss (1983), but it is mentioned that a certain quantity of pollen and/or brood is added together with honey to make an intoxicating beverage which has cultural significance. No volumes are given other than that wild beehives are eradicated due to high demand for brewing materials close to urban areas.

In the Kalahari (Kgalagadi) Trans-Frontier Park (Botswana-South Africa) the San people make an intoxicating beverage by mixing a little bit of honey, various herbs and sections of social weaver nests (E. McGregor pers.com). The social weaver nests are large grass structures containing many individual birds nests and a considerable amount of dry, grain-filled dung.

In Zambia 'sweet beer' is produced by mixing maize, mead, water, honey and the root of *Rhynchosia insignis* to produce beverages known as *mulava* or *mulaba*, depending on area (Forestry Department Report 1992). *Rhynchosia insignis* is a perennial herbaceous shrub, the roots of which are an important wild-gathered food in Zambia (Blay 2002). Again, attention must be drawn to the fact that Zambia also falls within the ancient Khoi-San region and that roots are added to the mead mixture.

The majority of Zambian honey is used to make the stronger *wala wa ndoka* or *wala wa kasolu* meads (Forestry Department Report 1992). The serving of these beverages at social gatherings is important and raises the beekeeper's status. Mead is used as currency for barter. An important part of the brewing process is the addition of brood and pollen to the brewing mixture which is placed in a calabash (Forestry Department Report 1992). A particularly interesting description of the actual fermentation is as follows, "The crop is crushed, mixed with water, and filled into the brewer's calabash where the brew will mature, bubbling and squeaking, within a few hours time." (Forestry Department Report 1992, p.39).

It is evident from these descriptions that certain of the meads described used adjuncts to facilitate fermentation. The use of loofa in Kenya and plant roots in Zambia is similar to the use of plant roots in South Africa. The scientific reasons for using the roots have not been clearly analysed, but it appears from descriptions in the literature discussed so far that they form an important part of the process as a source of yeast, or as a means of retaining yeast.

Figure 1.7 shows a composite map of *iQhilika/Kari* making reports, mead making reports and anthropological data concerning the Khoi-San. There appears to be a close correlation between the use of adjuncts to initiate fermentation and the historical presence of the Khoi-San. This supports the hypothesis that the use of adjuncts, specifically fibrous plant components, to initiate fermentation is based on an ancient Khoi-San fermentation system.

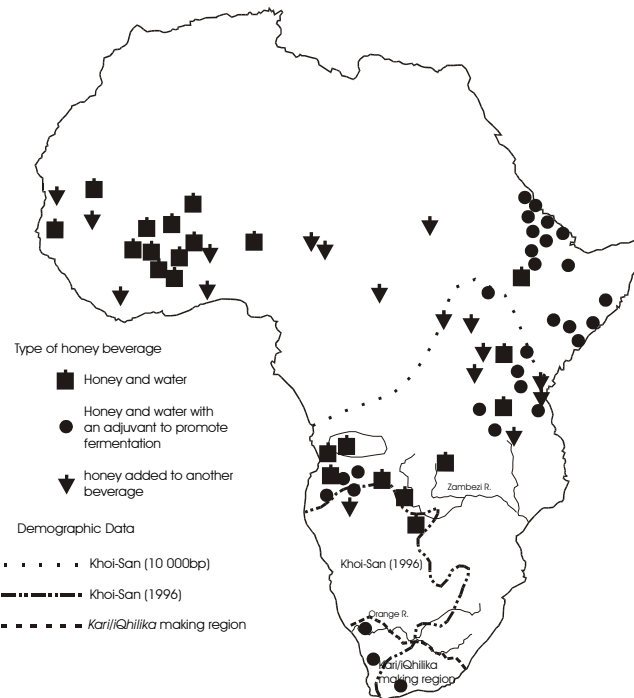


Figure 1. 7 A map compiled from linguistic information (Grobler *et al.* 1991, Comrie *et al.* 1996) and reports concerning *kari* and *iQhilika* distribution (Juritz 1906, Green 1949), and honey beverages (Crane 1999). It is evident that the present *karil/Qhilika* making region, which is now dominated by Xhosa, Afrikaans and English in that order (Grobler *et al.* 1991), falls within the historical Khoi-San region, as do the majority of the honey beverages recorded which use adjuvants to initiate fermentation in Africa.

1.1.7 The Eastern Cape as multi-cultural refuge of Khoi-San mead making

The Khoi-San have largely disappeared as a group who speak a Khoi-San language (Comrie *et al.* 1996). In the Eastern Cape of South Africa the Khoi-San descendants are Afrikaans speaking in the West and Xhosa speaking in the East (Grobler *et al.* 1991). In both cases, these descendants are the product of intermarriage between Khoi-San and Afrikaans or Xhosa people (Comrie *et al.* 1996). As a consequence the Eastern Cape represents an area where Khoi-San traditional knowledge is preserved in two neighbouring cultures which speak entirely unrelated languages, with Afrikaans being European in origin and Xhosa being Bantu in origin (Comrie *et al.* 1996). Both languages have however been influenced by the Khoi-San (Comrie *et al.* 1996).

From a research perspective, this region represents a favourable point to study the legacy of Khoi-San mead making.

1.1.8 Commercialisation of mead starting with *iQhilika*

In Europe, mead making has declined to such an extent that traditional mead making does not have the critical mass to support the transmission of a vibrant traditional knowledge system that would reflect the practises of mead makers during the height of mead production in the region. As a consequence, the scientific elucidation of current traditional European mead making practises is likely to yield a fraction of information that could have been elucidated in the Middle Ages using the equipment and strategies presently available to science. The scientific elucidation of traditional European mead making systems is consequently unlikely to produce sufficient data to form a foundation for the successful commercialisation of mead. This is evident in the slow growth of the current commercial mead industry despite recent improvements in production technology (e.g. Steinkraus & Morse 1966, Kime *et al.* 1991 a & b).

If mead is to advance, it is important to use a viable traditional mead making industry to perform a careful scientific analysis of the systems used in this industry. From this analysis, the fundamental biotechnological elements of the process could be used to build a sound biotechnological foundation for a commercial global mead industry. In this way mead would make the transition from a traditional to a commercial beverage as wine and beer have. The industry would then generate profits and this would encourage both state and private participation in research and development allowing the new commercial mead industry to remain competitive in much the same way the wine and beer industries do.

From a mead making perspective, the African traditional mead makers probably represent the single largest repository of mead making traditional knowledge on the planet. Africa therefore represents the logical starting point

for a scientific elucidation of traditional mead making to form the basis of a commercial mead industry.

The Khoi-San represent arguably the most ancient mead making cultural group in Africa and the Eastern Cape province of South Africa represents one of the largest concentrations of people with IKS derived from the Khoi-San. The Eastern Cape of South Africa therefore represents the logical starting point to begin researching the African mead, *iQhilika*, with the aim of commercialising this mead and setting a precedent for the commercialisation and preservation of the other unique meads of Africa.

The result of such commercialisation would see *iQhilika* merge into the global economy as a regionally unique product constructed on the foundation of regionally unique IKS, much the same way that Champagne is made in France or Sake in Japan. The *iQhilika* industry would pay its taxes to the South African government, encouraging further research and development into the product. This would ensure the long term survival of *iQhilika*. This would set a precedent to other African nations to commercialise their traditional mead industries so as to preserve them and turn them into revenue generators with long term growth and employment creation potential. Unlike any other alcohol producing industry, the production of mead making ingredients does not encourage environmental destruction through field agriculture and consequently provides an opportunity for sustainable growth.

1.2 Research hypothesis

The commercialisation of *iQhilika* may be based on both an understanding of the social functions of the beverage, its production and the scientific elucidation of the underlying traditional biotechnological principles.

1.3 Research objectives

- 1) To develop a scientific understanding of the social and traditional biotechnological production parameters of the traditional Xhosa/Khoi-San mead, *iQhilika/Kari*, and to employ these to develop a commercial *iQhilika/Kari* production system.
- 2) To investigate the microbiology and fermentation characteristics of the traditional *iQhilika* fermentation.
- 3) To develop an industrial *iQhilika* production system based on the investigation of the traditional fermentation.
- 4) To implement and evaluate a bioreactor design for bench-scale and production-scale *iQhilika* manufacture.

Chapter 2

The traditional *iQhilika* production system

2.1 Introduction

In Chapter 1 it was noted that in *iQhilika* production the roots of *Trichodiadema sp.* are used as an adjuvant in conjunction with honey and either pollen or bee brood to produce an alcoholic beverage which is culturally significant to both the Xhosa and Khoi-San people. The possible Khoi-San origin of adjuvant usage in African mead production has been argued. The Eastern Cape Province of South Africa has been shown to be an area with surviving Khoi-San influences, and hence a potentially important source of mead making IKS.

In order to provide an initial characterisation of the *iQhilika* production system, it would be important to understand *iQhilika* production in this region and to determine the methodologies used in making the beverage. An understanding of the culture surrounding *iQhilika* and its economic significance to this culture would also be important in placing the beverage into a social context. As a result, a survey of traditional mead production was undertaken in the Eastern Cape to establish an IKS baseline for the traditional mead production system.

2.2 A survey of *iQhilika* production in the Eastern Cape

An area approximately 500 km across and 350 km deep ranging from Kareedouw to East London in the Eastern Cape province of South Africa (Figure 2.1) was surveyed over a period of 8 years to gather information relating to traditional knowledge in *iQhilika* production. The nature of this knowledge and the attitude of suspicion of those that possess it is such that a direct structured survey would yield little data, hence the survey was conducted largely by oral interviews with traditional mead makers, or those that knew of traditional mead makers. Beekeeping work frequently facilitated contacts with traditional mead makers. By working bees without protection, a level of trust may be established between the observer with traditional knowledge and the beekeeper, in this case the author of this thesis. As will be shown later, in Xhosa culture the bees are representatives of the ancestors, hence if

a beekeeper can work bees without protection, the ancestors are seen to respect the beekeeper.

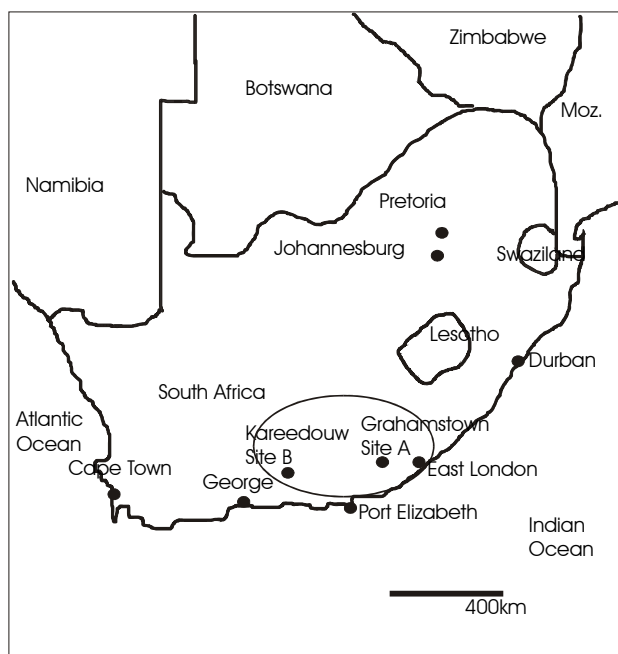


Figure 2. 1 The region in which *iQhilika* making in the Eastern Cape was surveyed (circle). Mead samples were obtained at the sites Grahamstown (A), which falls within the Xhosa *iQhilika* making region, and Kareedouw (B), which falls within the Khoi-San/Afrikaans *Kari* making region.

Traditional *iQhilika* makers were questioned as to the basic recipe they used, the identity of the plants used, the amount of honey/sugar added and whether other ingredients were used. The producers were also questioned as to the reasons for adding ingredients, and what could go wrong with the *iQhilika* production process. Other interesting anecdotes were also recorded where offered. Mead makers were asked where they thought *iQhilika* originally came from. A second angle of questioning dealt with the economic benefit derived from *iQhilika* production.

Where possible, recipes described by traditional brewers which involved the addition of fruit extracts, were tested when such seasonal fruits became available and the resulting beverages evaluated.

The results of this investigation are included in the following overview of the traditional practise.

2.2.1 The oral *iQhilika* tradition

In the area surrounding Grahamstown, two species of *Trichodiadema* are recorded at the Selmar Schonland Herbarium housed at the Albany Museum. These are *Trichodiadema intonsum* and *Trichodiadema stellatum* (Figure 2.2). Discussions with traditional brewers indicated that *T. intonsum* is called *imoela* but has the sub-description of being ‘*boesmans*’ which is an Afrikaans name for the San people, and *T. stellatum* is referred to as *imoela* without any descriptors, other than that it is not as good.



Figure 2. 2 *Trichodiadema stellatum* (A) and *Trichodiadema intonsum* (B). In the Makana region the *T. stellatum* is found in sandy grey soils whereas *T. intonsum* is found in clay and red soils near shale.

Discussions with traditional brewers throughout the Eastern Cape, revealed that the recipe used was highly similar throughout the region. The *Trichodiadema* roots are excavated, washed and peeled. The peeled roots are either cleaved diagonally or hammered lightly with a brick, then placed in slightly warm water for two days, following which the water is removed and replaced with fresh water. This is done partially to strengthen the yeast on the roots, and partially to remove the taste of the roots. The third wash contains a small quantity of honey and some extract of young bees (Xhosa - *amatyumsa*) and pollen (Xhosa - *intsinde*). The fourth wash consists of a stronger solution of the same. After this wash the roots are poured through a canvas bank bag and allowed to dry. At this point the root preparation is ready for brewing and is called ‘*imoela*’.

Brewing in the areas visited was always conducted in a 20 l or 50 l vessel which was filled with warm water and a mixture of honey and brood (1 cup of brood extract for the first 20 l batch and then for every second or third batch brewed thereafter) and if insufficient honey was available, brown sugar was added. The honey solution could, depending on availability be supplemented with juices of the fruits discussed later in this section. Fresh honeycombs, such as those shown by the beekeepers in Figure 2.3 are preferred.



Figure 2. 3 Beekeepers, Mr Vuyani Ntantiso (right) and Mr Phumlani Honi (left) show the fresh combs obtained from a wild beehive. These combs are prized by brewers as the honey is easy to squeeze from the combs.

The *Opuntia* cactus (prickly pear – English, *itolofiya* – Xhosa) is an invasive plant in the Eastern Cape, growing in thick stands and fruiting prolifically (Figure 2.4). The fruits are peeled and mashed then boiled and the extract poured through a sieve. The extract is further boiled and the scum that rises is skimmed off. The juice is mixed with brood and honey and fermented to make '*iQhilika ye tolofiya*'. Experimental batches produced in the lab were found to be pleasant but had an overpowering 'ester' taste.



Figure 2. 4 The prickly pear (*Opuntia ficus-indica*), an alien invasive from central America, produces copious sugary fruits which support a huge informal industry between February and March in the Makana region where pickers walk to the bush and harvest the fruits and sell them in Grahamstown. Bruised and damaged fruit are used for brewing.

The jacket plum, *Pappea capensis* (Figure 2.5), which grows from South Africa to Ethiopia (Venter & Venter 2002), produces small red litchi-like fruits known in Xhosa as *ilitshwe* which are used to make a type of *iQhilika*.



Figure 2. 5 The jacket plum, *Pappea capensis*. (Photographs J. Cambray)

The Kei-apple (*Dovyalis caffra*), which grows from the Eastern Cape to Malawi in central Africa, has fruits which are rich in malic acid (Venter & Venter 2002). In Figure 2.6 the heavy fruiting of the plant is evident. This fruit is used as part of the *iQhilika* mixture to produce a very strong beverage. The fruits of the closely related wild apricot (*Dovyalis zeyheri*), which ranges from the Eastern Cape to Zimbabwe (Venter & Venter 2002) are also used for *iQhilika* making.



Figure 2. 6 Kei-apple (*Dovyalis caffra*) fruits seen near the Matjana River, an area in Grahamstown where many *iQhilika* makers live.

In the coastal areas of the Eastern Cape an intensive pineapple industry has developed over the past 130 years. A large informal pineapple hawking industry sells to urban populations (Figure 2.7), and provides fruit to the informal brewing industry. The fruits of pineapples ferment rapidly on their own to give a weak pineapple ‘beer’, but if juiced, boiled and skimmed and added to *iQhilika*, they produce a drink of legendary intoxicating properties known in this region as ‘*pyna-pyna*’ or ‘*iQhilika ye pyna*’. Experimental batches of ‘*pyna-pyna*’ made in the lab were found to be unpleasantly acidic.



Figure 2. 7 A pineapple vendor on the streets of Grahamstown. Non-export, non-canning grade pineapples are sold off inexpensively to the fruit vendors by the ton. The vendors try to sell as much of the fruit as possible before it starts to spoil in the sun. The spoiled fruit is turned into ‘*pyna-pyna*’, a version of *iQhilika* made with pineapple.

In the Eastern Cape, apiculturists migrate thousands of hives to the 20 789 hectares of citrus groves in the province (Mabiletsa 2003) in September to produce citrus

honey. The pollen and nectar rich flowers encourage both honey and brood production. Many of the hives are raided by *iQhilika* makers for brewing ingredients. The honey and brood are mixed with the citrus fruits which ripen concurrently with flowering. It is reported by citrus farmers that specifically the overripe fruit of oranges and tangerines are juiced and used to make *iQhilika*. Experimental batches of citrus *iQhilika* produced in the lab were found to be pleasant but very acidic.

The wild plum, *Harpephyllum caffrum*, known as *umgwenya* in Xhosa, occurs between the Eastern Cape and northern Zimbabwe (Venter & Venter 2002). It is used in conjunction with honey to make a tangy *iQhilika*. Both the fruits of planted and wild trees are harvested for brewing *iQhilika* (Figure 2.8).



Figure 2. 8 A Xhosa women picks ‘umgwenya’ (*Harpephyllum caffrum*) fruit. Many streets in Grahamstown are lined with these trees due to their hardy and attractive nature.

Carissa macrocarpa (Figure 2.9), characterised by large fleshy fruits (Venter & Venter 2002), are used in *iQhilika* making. Experimental batches of *iQhilika* prepared in the lab using these fruits were pleasant and fruity.



Figure 2. 9 The large juicy fruits of *Carissa macrocarpa* are used to make a type of *iQhilika*.

Juritz (1906) mentions that in certain cases *iQhilika* was prepared with the addition of the bark or leaves of the 'umkwenkwe' tree. The possibility exists that this tree was *Rhamnus prinoides*.

Consultation with Mr Cecil Nonqane, a respected scholar of traditional Xhosa culture at the Albany Museum in Grahamstown and Mr Tony Dold, a Botanist in the Selmar Schonland Herbarium, showed that the *umkwenkwe* tree was *Pittisporum viridiflorum*, a tree with a range from South Africa to India (Venter & Venter 2002). Venter & Venter (2002) report that in South Africa the roots and bark of this tree are ground and added to beer as an aphrodisiac. Mr Nonqane was however doubtful of the benefits of adding this to *iQhilika* as it is an emetic.

Traditional *iQhilika* varies in sourness depending on the competence of the brewer and is generally a pleasant tasting beverage in rural areas. It has a woody, yeasty flavour with a ginger beer like quality. The appearance ranges from orange to light yellow and is slightly opaque - it is advisable to consume traditional *iQhilika* slowly as it can rapidly intoxicate an unacquainted drinker.

2.2.2 The origin of *iQhilika* according to oral history

Elderly Xhosa people in the rural areas of the Eastern Cape provided various stories of how *iQhilika* was invented. One elder explained that the *imoela* roots are particularly foul tasting, but are eaten as a food during lean times. He hypothesised that to make the roots taste better someone mixed them with honey and noticed that

the leftovers became intoxicating the next day. During lean times, beehives contain little honey, and this honey is often mixed with brood and pollen. Hence mixing the contents of a weak starving beehive with some roots during a drought could very well have yielded the first *iQhilika*. This accidental *iQhilika* making would however have occurred in the distant past as noted in Chapter 1.

The town of Kareedouw is in the predominantly Afrikaans speaking region of Khoi-San descendants in the Langkloof region of the Eastern Cape. Nienaber & Raper (1983) suggest that the name of Kareedouw was probably derived from that of a Khoi-San tribe.

The following suggestion as to the origin of the name of Kareedouw has been made by people in the area. The Outeniqua were another Khoi-San tribe (Nienaber & Raper 1983) and their name means 'honeygatherers'. The Outeniqua mountain range, named after its inhabitants, forms a barrier between Kareedouw and the sea. The Kouga mountains form a barrier in the opposite direction between Kareedouw and the arid interior, and a river capture incident has eroded a gorge through the these mountains (Cambray 1992). The gorge is lined with caves where large numbers of ancient sea shells and ostrich egg fragments have been found. This is noteworthy as the sea is two days walk (over the Outeniqua mountain range) from this point, and ostriches are found in the dry interior two days walk in the opposite direction. Communications with elderly farmers in the area revealed that the present day 'Kareedouw *Kari* Festival', a big mead drinking festival, is a remnant of the ancient annual coming together of the Khoi (such as the Outeniqua) from the coast and the San from the interior for trade and intermarriage between the tribes. The name Karee¹ means honey beer tree (Figure 2.10), and the original, as well as the present day, *Kari* festival coincides with the end of the flowering of this tree. *Kari* (*iQhilika*) was consumed at this festival as it still is today. The *kari* roots obtained from plants such as *Trichodiadema intonsum*, are from desert succulents. The rainforest covered Outeniqua mountains are not a favourable growth area for these plants, but the area is rich in honey. It is likely that this annual festival saw a trade of

¹ Dr J. Skead is currently 91 and has made a concerted effort to record the oral history of the Eastern Cape and placed this data in collections held at various museums thus preserving it for future generations. His file on 'iQhilika' was the starting point for this thesis. (www.epherald.co.za/colarc/elea/el161003.htm provides a brief description of his impact)

roots for honey, with the San bringing roots from the interior and the Khoi bringing honey from the coast.



Figure 2. 10 The Karee tree (*Rhus lancea*) which flowers from June – September (Venter & Venter 2002) is found throughout the Eastern Cape and the interior of South Africa. The karee tree produces an important honey flow which can yield the first crop of spring.

The explanations regarding the origin of the name of the town Kareedouw are backed up by the following information.

A report compiled by Francois Valentyn in 1726, concerning his exploration of the indigenous forests in the Outeniqua region, mentions that the San were found carrying honey in antelope skin bags over a pass in the Outeniqua mountains with the intention of bartering it with the Khoi (Crane 1999). Valentyn however described Outeniqua to be the Khoi word for ‘a man loaded with honey’ or ‘bags of the honey people’ (Crane 1999). Given the difficulty of translating languages as complex as the Khoi-San languages, it is probably only really safe to say that there was some association with honey.

2.2.3 Economic significance of *iQhilika*

Within Grahamstown three types of traditional brewers exist, those who make *iQhilika* on a daily basis (Figure 2.11), typically 20l /day; those who make a batch every Friday for sale over the weekend; and those who make *iQhilika* once a month for special events. A rough extrapolation based on the numbers of brewers in

Grahamstown suggested that the yearly *iQhilika* production would be in the region of 232 000 litres giving a per capita consumption of approximately 3.6 litres per annum.

Assuming a similar production ratio for the other towns within the *iQhilika* producing region of the Eastern Cape, which have a combined population of 1 725 100 people, according to the latest census data (Census 2001), the *iQhilika* production in the region would be 6.23 million litres per annum. This does not include rural populations who typically consume far higher volumes of *iQhilika*.



Figure 2. 11 Ms Zintombi Zabo, an *iQhilika* merchant, sells her mead together with a range of foods and cigarettes from a homemade shop front on the street. Her clients, low income, passing trade, drink the beverage on the spot from the container shown, for R5 a glass. Her busiest day is Monday when the drink is bought to steady hands after the weekend. Her biggest threat is police persecution when police empty her 20 l drum of mead into a nearby drain. Ms Zabo sells one 20 l drum on average every day. Her mead is contained in the yellow drum placed inconspicuously behind her table. The Coca-Cola bottle on the table is filled with home made ginger beer not *iQhilika*.

The questioning of *iQhilika* producers in Grahamstown showed that a considerable volume of *iQhilika* was produced and sold. For many people this represented a sole source of income. Most of the *iQhilika* merchants were single women supporting their families.

The standard price of *iQhilika* throughout the region is R 10-R 15 per litre. The theoretical value of *iQhilika* produced in the region would therefore be approximately R 62 000 000 which at an exchange rate of R 6.50/US\$ 1.00 and using the adjustment factor of Ong (1997), discussed in Chapter 1 gives an industry with a

value of US\$ 15 130 000 per annum. This represents an important unrecorded employment sector.

As a result of competition for the excise revenues derived from alcohol sales of products which compete with traditional *iQhilika*, the revenue services and police are the single largest threat to traditional brewers.

This is socially significant as the quality of *iQhilika* in the Makana region is probably far higher than that of the bulk wines. In this way the criminalisation of traditional *iQhilika* production deprives people of a livelihood option and damages the general health of the population, through forcing people to drink inferior beverages, and drains money from the local economy.

To quote Nelson Mandela discussing the gradual removal of liberties which characterised the onset of Apartheid in the 1950s (Mandela 1995, P. 173), "Every week we interviewed old women who brewed African beer as a way to supplement their tiny incomes, who now faced jail terms and fines they could not afford to pay." It is clear from this statement and data presented in this chapter that the advent of democracy has not yet changed the status of traditional mead makers.

2.2.4 Cultural significance of *iQhilika*

In Xhosa culture an important rite of passage is that of a boy becoming a man. The boy is circumcised (normally between 18-20 years of age) after which he spends a prolonged period of time in the bush wearing a loincloth and sleeping in a grass shelter. He is visited by the elders who impart skills and cultural knowledge. Ruling clans such as the *Madiba* and *Chowe* clans are reported to teach their initiates how to make *iQhilika*. At the end of the period in the bush, the boy is welcomed into the community as a man at an *umgidi* ceremony. He is expected to present gifts of alcohol to the elders of the community, which in the past consisted of *iQhilika*. However in present times anything from good brandies to whiskeys such as Johnnie Walker Red, Black or even Blue in wealthy circles, are acceptable.

Bees are believed by the Xhosa to be linked to the ancestors, hence bees are viewed as the bearers of many omens. One such omen, is the *livingane* or death's

head moth (Figure 2.12), *Acherontia atropos*, (Hepburn & Radloff 1998). The moth resides in hives and flies screeching loudly into the air when hives are raided for *iQhilika* making. If the moth is to strike a gatherer on the head it is believed that death by heart attack will follow rapidly.



Figure 2. 12 The death's head moth, so called because of the skull shape on its thorax. This particular moth screeched loudly when captured. (Photograph J. Cambray)

2.3 Analysis of a model *iQhilika* production system

2.3.1 Methods

2.3.1.1 Selecting a model traditional bioreactor to analyse

After observing many traditional bioreactors in operation, the system operated by Mr Mathews Makwana of Makana's Kop informal settlement was selected as a model system. Mr Makwana is highly regarded by his peers as a producer of quality *iQhilika* and his products are organoleptically consistent. The basic parameters of the bioreactor (Figure 2.13) were measured to determine total volume, liquid volume, and the volume displaced by root segments.



Figure 2. 13 The traditional bioreactor vessel of Mr Makwana.

The traditional *T.intonsum* root preparation was visually inspected to determine the surface characteristics.

Substrate was prepared according to the standard traditional *iQhilika* recipe (to be presented in results). The volumes and characteristics of ingredients were recorded.

2.3.1.2 Sampling

Liquid Samples

Samples (50 ml) of *iQhilika* were withdrawn and frozen at regular intervals as the fermentation progressed. Samples were taken from the top 2 cm of the bioreactor at various points during the fermentation (see Figure 2.19).

pH

The pH of samples was measured with a WTW Inolab pH meter.

Biomass and root analysis

Root samples were taken as fermentation commenced. Once the fermentation had reached a high level of activity, samples were again taken and when the fermentation was largely complete, final root samples were taken again. The roots were washed of all biomass with a 0.2 M NaCl solution, and the wash water filtered through pre-weighed 0.22 μ m filtration membranes (Whatman). The filtered samples

were rinsed with 10 ml deionised water to remove NaCl residues. Samples of liquid (2 ml) from the bioreactor were also taken and filtered. The dry weight of the biomass was measured after the 0.22 µm filtration membranes (Whatman) had been placed in an oven at 60°C until a steady weight was obtained. The volume of the representative root samples was determined by displacement in a 10 ml measuring cylinder. The surface area was determined by wrapping a thin adhesive thread around the root section at three points to determine circumference, which was compared to calculations using the diameter determined with a calliper, and then measuring the length of the root to calculate the surface area.

Liquid samples were taken using sterile 5 mm internal diameter glass pipes at depths of 1 cm, 15 cm, and 30 cm. The samples were taken at 6.5 hours (active fermentation) and 31 hours (less active fermentation). Samples were of 20 ml volume.

Samples for suspended biomass measurement were taken from the top 2 cm of the bioreactor at various points during the fermentation. Two millilitres of the liquid samples were filtered through 0.22 micron filtration membranes (Whatman) and dry biomass weights determined.

2.3.1.3 Alcohol and sugar analysis

HPLC Analysis

The liquid samples were filtered through a 0.22 micron cellulose acetate syringe filter (Cameo). The diluted samples were injected onto a Hamilton HC40 Ca⁺⁺ column with a length of 308 mm and a diameter of 7.8 mm consisting of 4% cross linked gel using size exclusion as the primary mode of separation with ligand exchange providing additional separation. The injector was fitted with a 10 µl injection loop. The system was operated at a flow rate of 0.6 ml/min provided by a Beckman 110B pump system, with water as the mobile phase and the column heated to 80°C in an HPLC Technologies column heater. The eluant was analysed with a Knauer Refractive Index detector. A standard solution was run every 10 runs to detect deviations. Data was captured using 32 Karat Software (version 3.0 Beckman). Ethanol yield was calculated as ethanol yield per gram of glucose/fructose which have identical molecular weights. Sucrose, comprising a molecule of glucose and a

molecule of fructose is 5% lighter than the sum of the molecule weights of these sugars.

2.3.1.4 Electron microscopy

Root samples and floc samples were taken and fixed in 2.5% gluteraldehyde solution in 0.1 M phosphate buffer (pH 7.0) overnight at 4°C. The following day samples were washed twice in 0.1M phosphate buffer for 10 min. The samples were then alcohol dehydrated by soaking in 30%, 50%, 60%, 80%, 90% and 100% ethanol each for 10 minutes. The samples were additionally washed in another two 100% ethanol rinses. The samples was then soaked in a 75:25 ethanol/amyl acetate mixture for 15-20 minutes, followed by 15-20 minutes in a 50:50 ethanol/amyl acetate solution and finally 15-20 minutes in 25:75 ethanol/amyl acetate solution. The samples were then taken and soaked in pure amyl acetate for 20 minutes. A Polaron E300 Critical Drying Apparatus was used to dry the samples following which they were sputter coated with gold in a Polaron E5100 Sputter Coating Unit. Prepared samples were visualized on a JEOL JSM 840 SEM.

2.4 Results

2.4.1 Analysis of a model *iQhilika* bioreactor

2.4.1.1 Bioreactor vessel

The bioreactor consisted of a bucket with a radius of 13.5 cm, a working depth of 30 cm and a working volume of 17-18 l (Figure 2.13). The bioreactor had a total volume of 20 l. Roots of *Trichodiadema intonsum* were used as traditional biomass supports. The average *T.intonsum* root section was determined to have a volume of 0.36 ml/cm (linear measurement performed with calliper and displacement determined in a measuring cylinder for 50 sections). The average surface area per centimetre was calculated to be 2 cm² (circumference measured with adhesive thread) giving a total root bed surface of approximately 1.4 m². The roots filled the bioreactor to the 4.3 litre mark and displaced a volume of 2.46 l.

While discussing the traditional bioreactor it was mentioned by Mr Makwana that he believed that before plastic buckets and paraffin drums were available for mead making purposes, that *iQhilika* was made in clay pots (*imbiza yedongwe*) and

calabashes (*iselwa*). In remote rural areas *iselwa* are still used due to their traditional importance.

2.4.2.2 Biomass

The inoculum used consisted of a cotton bag containing sufficient roots of *T.intonsum* to fill the bioreactor to the 12 cm mark. The biomass had been dried by hanging in a the bag under the eaves of Mr Makwana's house. The imoela is normally dried in this fashion between batches. The roots were covered in a dry caked layer with a strong smell of yeast. A small amount of filamentous fungus was evident on the surface of some of the roots (Figure 2.14). Certain patches appeared to be a *Penicillium sp.*, but the majority appeared to be an *Aspergillus sp.*



Figure 2. 14 The dry biomass preparation with filamentous fungi growing on the surface of the *Trichodiadema intonsum* roots.

2.4.2.3 Substrate preparation

A honey solution with a specific gravity of 1.055 was prepared and a sample withdrawn and frozen. 350 ml of extract of young bee brood/pollen combs was

added and the substrate was added to the bioreactor, as per normal traditional brewing procedures. The final working volume of the bioreactor was 18.48 l.

2.4.2.4 Biomass analysis

Analysis of the *T.intonsum* root sections showed that the roots acted as a support, immobilising yeast and bacteria. In Figure 2.15 it is evident that at the beginning of the fermentation the supports were spread with more at the bottom and as the fermentation became active the supports moved upwards, and then settled back to the bottom as the fermentation slowed.

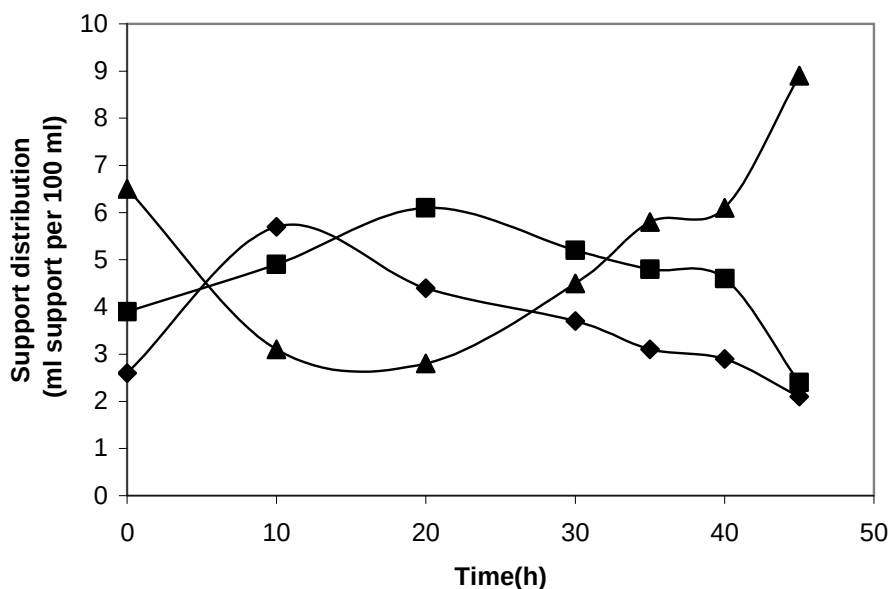


Figure 2. 15 The rough distribution of the *T.intonsum* supports between the top (◆), middle (■) and bottom (▲) over the duration of the fermentation. The supports displayed varying levels of buoyancy, but gradually settled to the base of the bioreactor as time progressed.

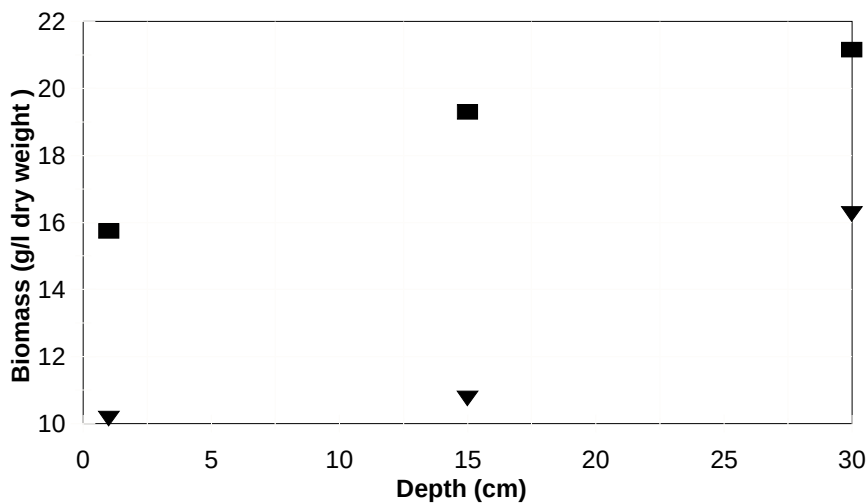


Figure 2. 16 The dry weight of freely suspended cells at three depths in the bioreactor after 6.5 hours (▼) and 31 hours (■) during the fermentation.

Figure 2.16 shows that after 6.5 hours of fermentation the non-root immobilised biomass was present in large volumes at the bottom of the bioreactor. After 31 hours the amount of freely suspended biomass at all levels in the bioreactor had increased, with proportionately less freely suspended biomass at the bottom of the bioreactor.

Figure 2.17 shows the estimated total dry weight of biomass immobilised in the bioreactor. The initial determination of the root surface area provided an estimated total surface area for biomass immobilisation. The root samples taken during the fermentation were stripped of biomass which was then strained through a small gauze to remove root fibres. The strained solution of cells and buffer was filtered and processed for dry weight determination. The surface area of the sections from which the biomass had been removed was calculated and this was used to estimate total biomass in the bioreactor. The largest concentration of immobilised biomass was evident at the start of the fermentation with a return to close this value at the end of the fermentation. It is likely that during the drying process, biomass will attach to root supports leading to a return to the initial value.

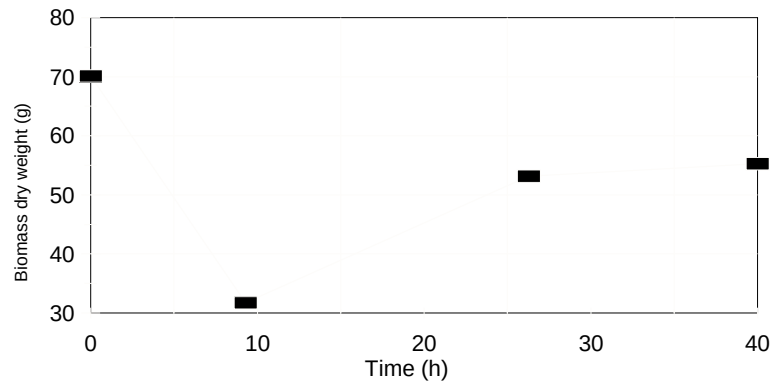


Figure 2. 17 Dry weight immobilised biomass profile over the duration of the fermentation. This was calculated by determining the biomass per cm^2 of root sample and then multiplying by the 1.4 m^2 surface of roots in the bioreactor.

2.4.2.5 Fermentation profiles

The general appearance of the surface of the bioreactor is shown in Figure 2.18. The extra-cellular polymeric substances in the *imoela* biomass preparation initially produced a foamy layer which covered the surface of the bioreactor. As the fermentation progressed this layer dissipated.



Figure 2. 18 The bioreactor after 2 hours of fermentation. Vigorous carbon dioxide gas evolution was evident. Note the hydrometer which was used to provide a rough indication of the rate of fermentation to allow appropriate biomass sample taking.

In Figure 2.19 the conversion of the honey sugars, fructose, glucose and sucrose to ethanol over time is shown, with fructose and glucose showing an inverse relationship to ethanol production.

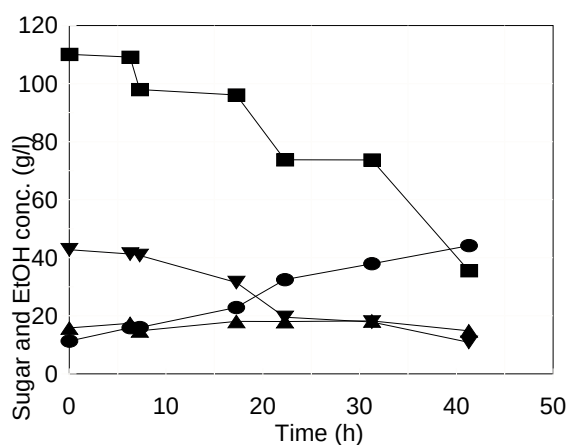


Figure 2. 19 Bioreactor sugar and ethanol concentrations over time for (■) Fructose, (▲) Sucrose, (●) Ethanol, (▼) Glucose.

Figure 2.20 shows an oscillation in the ethanol yield of the bioreactor.

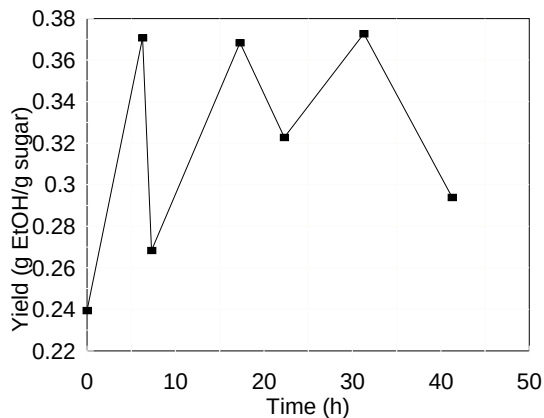


Figure 2. 20 The yield of the bioreactor over time calculated as g ethanol formed per g of sugar used.

Figure 2.21 and 2.22 show that although the bioreactor appeared evenly mixed, the yield of ethanol was highest in the middle of the bioreactor after 6.5 hours.

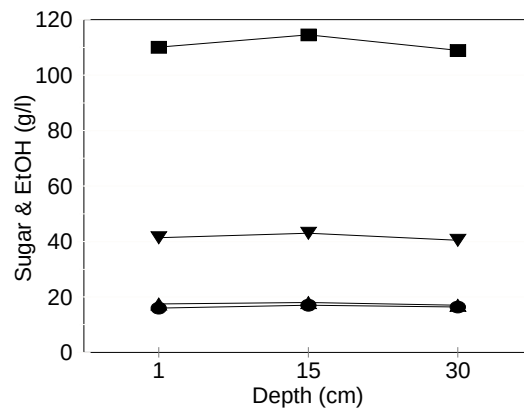


Figure 2. 21 The profile of the bioreactor after 6.5 hours for (■) Fructose, (▲) Sucrose, (●) Ethanol, (▼) Glucose.

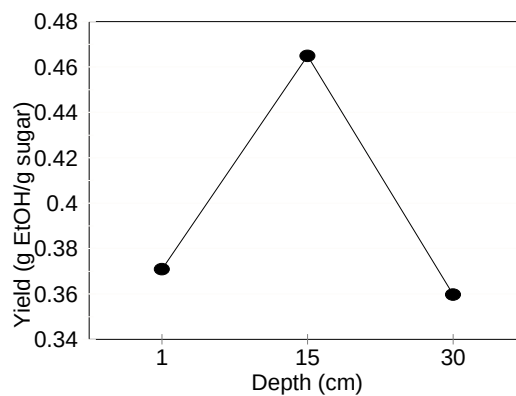


Figure 2. 22 The profile of the bioreactor in terms of Ethanol yield per gram of sugar utilized after 6.5 hours.

Figures 2.23 and 2.24 show that after 31 hours the conditions in the bioreactor had changed, with the system still well mixed, but the yield was highest close to upper surface which had contact with the atmosphere.

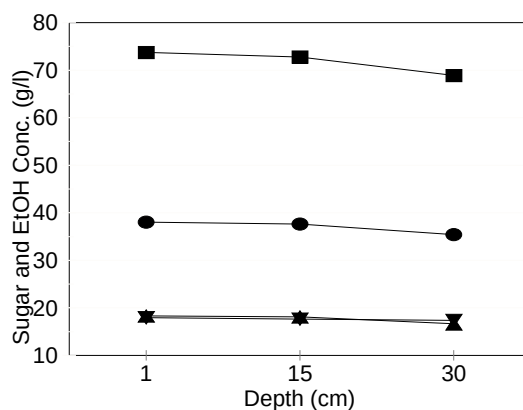


Figure 2. 23 The profile of the bioreactor after 31 hours for (■) Fructose, (▲) Sucrose, (●) Ethanol, (▼) Glucose.

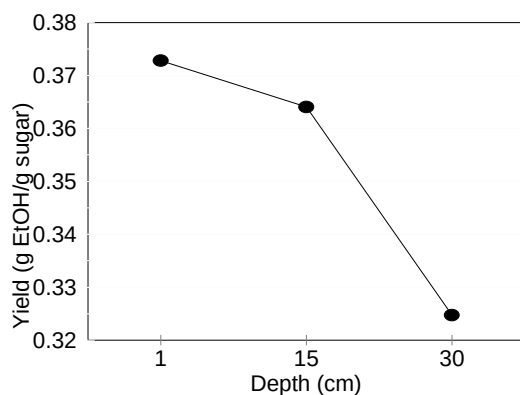


Figure 2. 24 The ethanol yield profile of the bioreactor after 31 hours.

The final alcohol content in the bioreactor was 44.25 g/l (7.3% by volume). The total volume of the bioreactor was 20 l and the fermentation took 43.5 hours. The bioreactor therefore had a volumetric productivity of 0.74 g ethanol per l of bioreactor per hour. The liquid volume of the bioreactor was 18.48 l. This gives a productivity for the liquid portion of the bioreactor of 0.8 g ethanol per l of liquid volume per hour.

Figure 2.25 shows that a gradual decrease in pH occurred over time. This was accompanied by an increasingly sour smell to the fermentation.

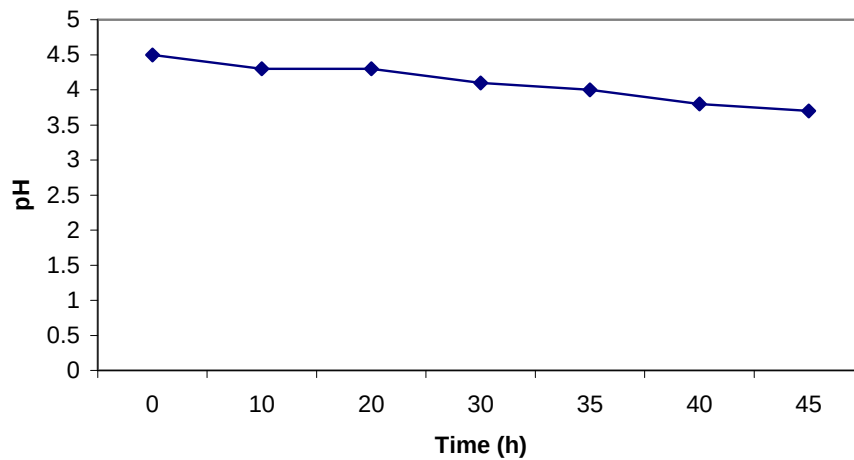


Figure 2. 25 Time profile of pH in the bioreactor.

2.4.2.6 Biomass and root analysis

SEM of the root supports showed the presence of yeast and an extra-cellular polymeric substance/mucoid matrix bacteria (Figure 2.26), crystals and a fission yeast (Figure 2.27). Flocs which formed without root supports (Figure 2.29) were found to contain yeast and bacteria held together by an extra-cellular polymeric substance (Figure 2.28).

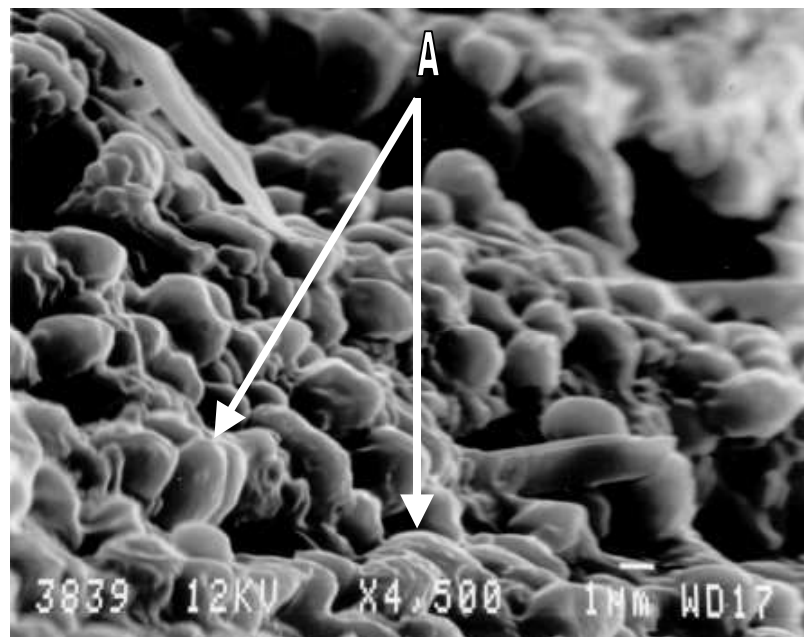


Figure 2. 26 SEM of the surface of a piece of *T.intonsum* root with biofilm. The roots used had been used to make many batches of *iQhilika* and had been dried and re-hydrated many times. Note the mucoid matrix (A) covering the yeast cells. This is similar to that observed by Vadasz *et al.* (2000) in wine yeasts.

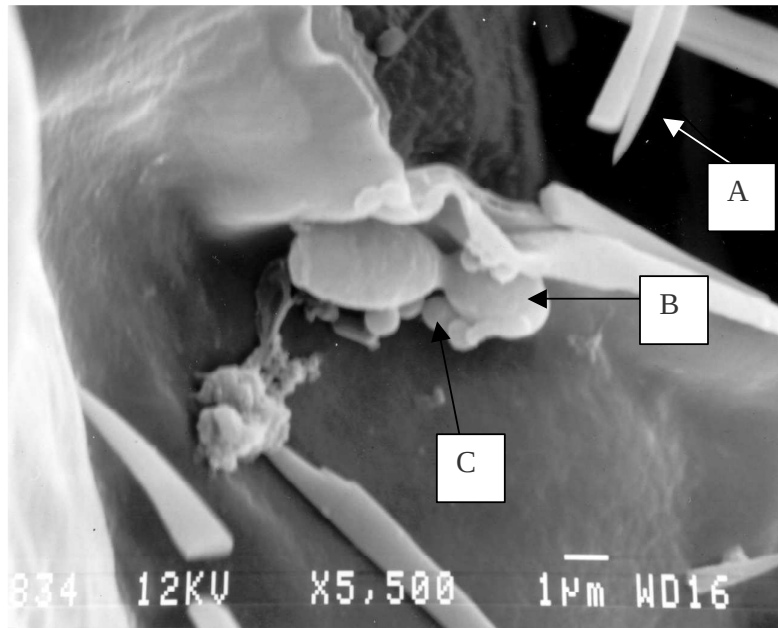


Figure 2. 27 An SEM showing the inside of a cavity within a *Trichodiadema* root. Insoluble crystals (A), a common feature of the roots, together with a fission yeast (B) and a few small cocci are visible (C). It is interesting to note fission yeast were not isolated from any *iQhilika* samples during this research project.

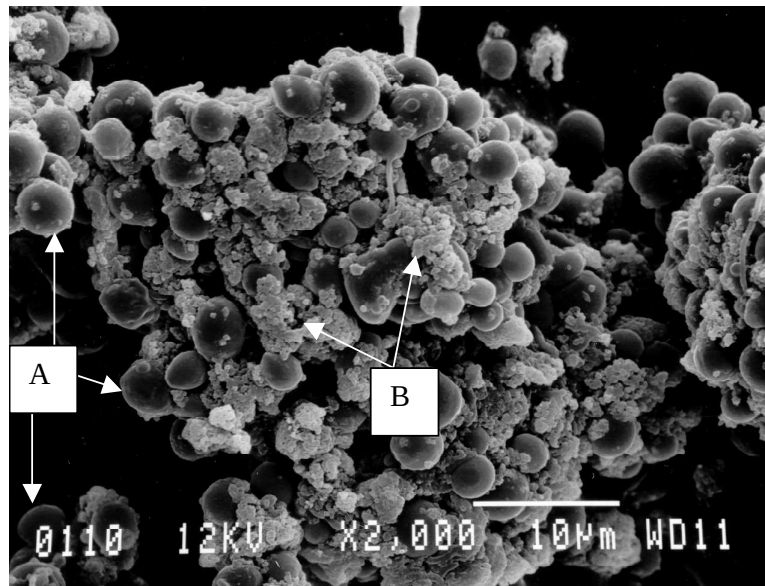


Figure 2. 28 An SEM of a floc which formed spontaneously without a *T.intonsum* support. The yeast cells are evident (A), as well as a large number of smaller bacterial cells and extracellular polymeric substances holding the floc together (B).

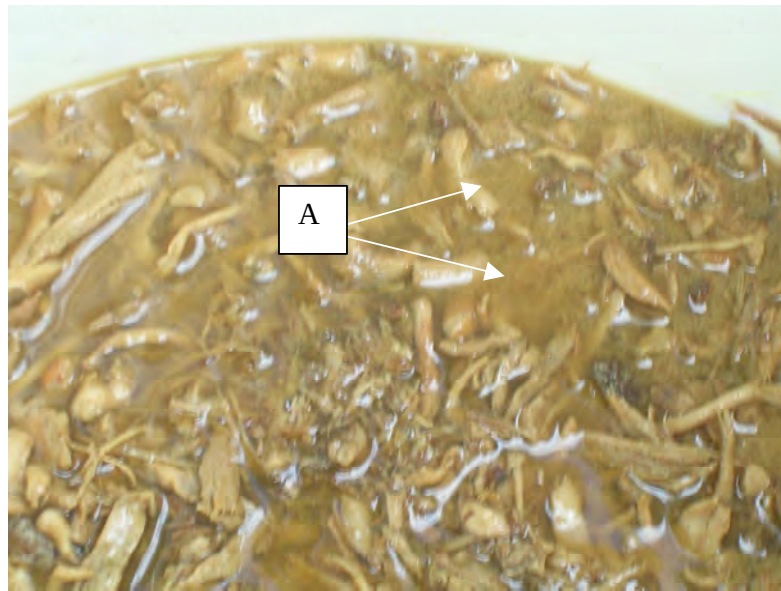


Figure 2. 29 The biomass preparation after draining of the bioreactor. The spontaneously flocculated yeast is evident in the spaces between roots.

2.5 Discussion

2.5.1 Biomass immobilisation

Scanning electron micrographs (SEM), as well as volumetric biomass analysis in the bioreactor revealed that the *T.intonsum* roots performed the role of a partial support for the biomass. The biomass immobilised on the roots was highest at the start of the fermentation – when the dry roots were mixed with the must. A large percentage of the immobilised biomass then became freely suspended and left the root supports, after which the root immobilised biomass gradually increased in volume as the fermentation progressed. The traditional process of straining biomass through a cloth at the end of fermentation and allowing it to dry results in the freely suspended floc attaching to the roots.

SEM micrographs revealed a large number of yeast and bacterial cells immobilised on the outside of the root supports by an extra-cellular polymeric layer (Figure 2.26). Internal entrapment within the structure of the roots was also evident (Figure 2.27). The origin of the extracellular polymeric layers is unknown, and may have been either bacterial, yeast derived or both. A small number of filamentous fungi were also evident – it is likely that these were not active during the fermentation, but were

rather remnants of fungi seen on the outside of the biomass preparation as a result of the drying phase.

SEM micrographs of flocs from the bioreactor revealed that these were also composite with yeast and bacteria conglomerated in the extra-cellular polymeric layer. In the case of the flocs, the majority of bacteria appear to be cocci, whereas in the biofilm community on the roots, a mixture of rods and cocci are evident.

Other interesting artefacts in the root support are the crystals – these appear to be insoluble (the root preparation had been used for many years) and form part of the root structure (Figure 2.27). They do not appear to play any role in the adhesion of either bacterial or yeast cells – suggesting that the means of adhesion used by the cells does not involve surfaces such as the crystals.

Kourkoutas *et al.* (2004) review the use of immobilisation technologies suitable for use in beverage and potable alcohol production. They list four basic immobilisation strategies, immobilization on solid carrier surfaces, entrapment within a porous matrix, cell flocculation and mechanical containment behind a barrier.

The *Trichodiadema intonsum* biomass preparation acts as a solid carrier surface, although small holes in its surface act as a porous matrix. A certain percentage of the biomass in the preparation consists of flocs without a *T.intonsum* core. Depending on the point of the fermentation this ranged from 10-30% as determined by visual inspection.

The operating conditions of the traditional *iQhilika* bioreactor are characterised by moderately high sugar content, periodically elevated ethanol concentrations of 7% (by volume) and removal of partially fermented product for consumption. These conditions are likely to enrich for moderately alcohol tolerant, flocculating, osmotolerant yeasts in much the same way that Sree *et al.* (2000) isolated strains of yeast with these characteristics. In the traditional *iQhilika* bioreactor, at the point when the honey medium is added, the moderately high sugar content would have a selective pressure on those cells able to grow at that osmotic potential. As the fermentation progresses, the sugars are converted to alcohol, which has a similar selective pressure against non-alcohol tolerant cells. The alcohol content is not high,

but fermentation above 4% alcohol generally selects for *Saccharomyces* yeasts (Pretorius 2000). The cells best able to ferment and flocculate will be retained by the system.

Bowen *et al.* (2001) used atomic force microscopy to measure the adhesion of *Saccharomyces cerevisiae* to various surfaces. Stationary phase cells were found to show the strongest adhesion to a mica surface. Many immobilised cell systems report reduced cell growth rate and a pseudostationary phase (Royston 1966), which would consequently encourage cell to cell and cell to matrix adhesion.

Kourkoutas *et al.* (2004) further review the basic parameters that an immobilization matrix should meet for use in beverage production as follows:

- 1) It should have a large functional group rich surface for attachment.
- 2) It should be easily handled and regenerated.
- 3) Biomass must be stable and maintain high cell viability for long time periods.
- 4) The biological activity of the cells should not be harmed by the immobilisation process.
- 5) The porosity of the support should be consistent allowing effective exchange of substrate, metabolites and cofactors.
- 6) The carrier should be mechanically, chemically, biologically and thermally stable so that it is not easily degraded by factors in the reactor.
- 7) The carrier should be cost effective to produce and its use should be scalable.
- 8) The support should pose no threat to the consumer, either real or perceived.
- 9)

A number of supports used by workers contain substances which may elicit negative public responses due to health or religious reasons. Bardi *et al.* (1997) used gluten pellets to immobilise wine yeasts and Plessas *et al.* (2005) used a starch-gluten milk matrix to a similar end. Gluten is a protein in *Triticales* (e.g. wheat) to which a certain percentage of the population show extreme sensitivity in the form of Coeliac disease (Anderton 2004). The use of materials such as gelatin (de Alteriis *et al.* 2001) and collagen as reviewed by Kourkoutas *et al.* (2004), will exclude Kosher individuals

(Eliasi & Dwyer 2002), and possibly some Hindu people who may consume alcohol. If the products of fermentation are used to make vinegar, other than wine vinegar, which is prohibited by Muslim law (Eliasi & Dwyer 2002), they may also be rejected by Muslim people.

Stainless steel is used widely during the processing of most food products and is perceived as a safe product by the market. Bekers *et al.* (1999) made use of stainless steel wire spheres to immobilise yeast in this way using an support that is perceived as safe.

Guenette & Duvnjak (1996) used *S. cerevisiae* ATCC 39859 immobilised on wood blocks to remove glucose from high fructose corn syrup and convert it to alcohol, with the result that the effluent contained a fructose and ethanol solution which could be separated and sold. The immobilisation of the yeast on wood blocks led to a dramatic improvement over results they had obtained in a previous experiment using alginate immobilised yeast. A wood block is generally regarded as safe, especially if it is an oak block or other wood type already used in food and beverage processing.

Natural materials with large surface areas have been explored as supports for *S.cerevisiae* by researchers. Examples include loofa sponge (Ogbonna *et al.* 2001) and jute fabric (D'Souza & Melo, 2001).

Kourkoutas *et al.* (2002 a & b, 2003) in a series of papers on fruit cut immobilised yeast for wine making in both batch and continuous systems employed the fact that fruits such as apples and quinces are well known and entirely food grade, being themselves common foods. These fruits are not excluded by any religious groups. The fruits are inexpensive, and effective and their use produced acceptable wines.

Many remedies containing medicinal herbs, which are not even known by a target market, are regarded as safe because they are natural. This has led to an explosive growth in herbal remedies (Miller *et al.* 2004). Human beings display neophobia, a fear of new things, and are more likely to consume a product if somebody else has done so before (Frewer *et al.* 2001). The fact that specific herbs have been used for a long time by many people helps the public feel at ease and the herb is generally regarded as safe. This is not always however true, as is seen by the fact that both

potatoes and avocado pears, which are generally regarded as safe, are in fact slightly toxic (van Wyk *et al.* 2002). In the case of *iQhilika*, and specifically the roots of *Trichodiadema intonsum*, the widespread and ancient use of the plant can give it generally regarded as safe status (GRAS status).

Hence the processing advantages of advanced immobilisation systems such as those reviewed by Kourkoutas *et al.* (2004) can be obtained using the traditional *iQhilika* immobilisation material, *T.intonsum* roots which due to their ancient historical use are generally regarded as safe. In this way a high ethanol yield can be expected as most of the active biomass is retained from batch to batch. The disadvantage however of using immobilised biomass in the traditional *iQhilika* bioreactor is that both the beneficial and the undesirable microbes become trapped in the *imoela* root preparation.

In the preparation of *T.intonsum* roots for *iQhilika* making, the roots are peeled and soaked in water repeatedly and the water poured off. The roots of a specific brewer may be used for up to ten years with repeated drying and are not prone to disintegration. The roots shown in this Chapter were from an *imoela* preparation which had been used for this period of time. After ten years of weekly use it is reasonable to conclude that the only function of the roots will be as an inert immobilisation support and that the roots themselves contribute little flavour to the product. After long periods of use the roots retain their ability to function as required in the traditional bioreactor.

It appears that the dominant organoleptic properties are derived from the honey and bee ingredients and the action thereupon of the *Trichodiadema* immobilised biomass.

2.5.2 Bioreactor operation

Upon adding media to the bioreactor, the *T.intonsum* root support immediately suspended itself throughout the bioreactor in a reasonably even packing (Figure 2.15). As the fermentation progressed the roots gradually settled, although some roots were observed to cycle between the bottom and top of the bioreactor. The roots are traditionally dried between brewing, and consequently any water in the roots is largely removed by drying. The roots, when added to the bioreactor, float

and gradually sink. Gas generation from yeasts immobilised within the root structures appears to cause the roots to maintain their buoyancy until the fermentation slows, at which point the roots settle. The formation of gas bubbles within a support can be advantageous in terms of the buoyancy, but can also create dead spots where no fermentation occurs due to occlusion of nutrients by a bubble (Jamai *et al.* 2001).

Traditional brewers explained that if the roots are used frequently and not dried adequately, they will settle into the base of the bioreactor and not float. There was no mention as to whether this was undesirable, other than that the *iQhilika* produced is reportedly less strong.

A gradual decrease in pH from pH 4.5 to pH 3.7 was evident (Figure 2.25). This decline was accompanied by an increasingly sour smell in the bioreactor. Traditional brewers monitor this sourness and dry the root preparation if it becomes excessive. This suggests a gradual increase in spoilage organisms. The mode of inactivation of the spoilage organisms is of interest, but not dealt with in this thesis. It may be possible that the growth of mould on the root surface results in the production of inhibitory products which kill bacteria but not yeast. It may also be possible that the spoilage organisms are forced to go dormant and hence drying allows the yeast, which becomes active very rapidly to complete the fermentation without too much interference from spoilage organisms.

It is evident from Figure 2.19 that once glucose was largely exhausted, fructose was more rapidly utilized. Slower fermentation of fructose over glucose has been observed in many wine yeast strains (Berthels *et al.* 2004). Figure 2.20 shows a reasonably wide range of ethanol yields over time – this is possibly due to synchronised growth of the cells, with periods of cell growth having been reported to give lower ethanol yields (Beuse *et al.* 1998).

The depth profiles (Figure 2.21, 2.22, 2.23, 2.24) of the bioreactor at 6.5 hours and 31 hours show an interesting result that initially ethanol yield is highest in the middle of the bioreactor, but with time its yield becomes highest at the surface. It is likely that this is due to the presence of oxygen near the surface which allowed cells to maintain a higher viability due to synthesis of medium chain fatty acids. As the

ethanol concentration increases, these fatty acids become more significant in maintaining cell viability. This phenomenon has been reported for *Saccharomyces cerevisiae* (Bardi *et al.* 1999, Gil *et al.* 1990). In breweries, yeast growth is stimulated prior to pitching of yeast by aeration to enable sterol production (Lieberman 1980).

2.5.3 Bioreactor classification

Bioreactors can be classified in many ways. Atkinson & Mavituna (1991) list four linked systems of classification:

- 1) The mode of substrate addition and bioreactor geometry, giving batch; continuous; tubular packed bed; and tubular fluidised bed reactors.
- 2) The configuration of biomass within the reactor, either freely suspended or immobilised.
- 3) The mode providing mixing, such as mechanical, gas and external agitation.
- 4) The type of biocatalyst, enzyme, aerobic micro-organism or anaerobic micro-organism.

Each of these systems can be divided into more descriptive sub categories, hence batch reactors could be operated as batch, or fed batch (Moo-Young *et al.* 1985). A continuous reactor with immobilised biomass may perform without mixing giving a plug flow bioreactor (Moo-Young *et al.* 1985).

In the case of the traditional *iQhilika* bioreactor the biomass preparation is re-used, but is separated from the media post fermentation and the majority of the biomass is retained for re-use. The fermentation is allowed to run its course before consumption of the product. The traditional *iQhilika* bioreactor is hence a sequential batch system as the biomass is returned and used for subsequent batches.

When the *iQhilika* bioreactor is inoculated, the majority² (± 70 %) of the biomass is immobilised on the *T.intonsum* root support (*imoela*), or in flocs (± 30 %), hence the traditional *iQhilika* bioreactor uses immobilised yeast.

² As determined by visual inspection.

The traditional *iQhilika* bioreactor was initially a well mixed system with gas bubbles generated by fermentation providing pneumatic mixing. As the fermentation progressed this mixing became slower and less effective and stratification was encountered leading to a non-homogenous bioreactor.

The *iQhilika* bioreactor is an anaerobic immobilised biomass system. The slightly higher yield near the surface, where contact with the atmosphere occurred, suggests that although largely anaerobic, oxygen plays a role in maintaining the fermentative efficiency of the biomass.

The reactor can thus be classified as a non-homogenous anaerobic batch tank bioreactor (operated sequentially), with predominantly immobilised biomass.

2.6 Conclusion

The *iQhilika* production process did not vary greatly between the Xhosa and Afrikaans speaking regions in which it is found. The culture and mythology surrounding the beverage appeared to result in accurate transmission of the recipe and traditional biotechnological systems, which in turn results in the production of a beverage of a high level of organoleptic similarity throughout the region in which it is produced.

Analysis of a model *iQhilika* bioreactor allowed it to be classified as a non-homogenous anaerobic batch tank bioreactor (operated sequentially), with predominantly immobilised yeast that converts honey sugars to alcohol and carbon dioxide. It is operated in a sequential batch configuration with biomass retained between batches. A small amount of oxygen is absorbed by yeast cells at the air/*iQhilika* interface leading to increased efficiency of cells in this region as ethanol concentrations increase. The dominant organoleptic properties of the beverage are produced by the action of the immobilised biomass on the diluted honey and bee products.

The traditional *iQhilika* making process represents a biotechnologically more advanced fermentation than any of the traditional and commercial mead production systems reviewed in the literature. A natural source of nutrients (pollen and/or brood)

and an efficient biomass immobilisation system results in a fermentation which achieves 7 % alcohol by volume in 43.5 hours. This is in stark contrast to commercial mead making systems which achieve similar results in far longer periods of time. Hence it is safe to conclude that *iQhilika* is not a primitive African mead but is in fact a traditional process which employs technologies, such as biomass immobilisation, which are relatively recent concepts in scientific process design.

Chapter 3

Characterisation of the dominant *iQhilika* mycoflora

3.1 Introduction

3.1.1 Spontaneous fermentations

Traditional fermentation processes in Europe relied on spontaneous fermentations caused by the presence of yeast on improperly cleaned beer fermentation equipment (Hough 1984) or from the skins of grapes in the case of wines (Ferraro *et al.* 2000, Pretorius 2000, Rementeria *et al.* 2003, Clemente-Jimenez *et al.* 2004). Sanni *et al.* (1999), in a report on microbial deterioration in fermented alcoholic beverages in Nigeria sum-up the dangers of spontaneous fermentations as follows; “The production of these beverages like any other traditional fermented foods in Africa is by the age-long method of chance inoculation and uncontrolled fermentation process. Therefore, the usual variations in quality and stability of the products are not unexpected.”

3.1.2 The ecology of spontaneous wine fermentations

The conversion of grape must to wine in traditional fermentations is the product of a complex series of fermentations and interactions between many microbes present in the fermentation environment (Esteve-Zarzoso *et al.* 1998, Ferraro *et al.* 2000, Pretorius 2000, Clemente-Jimenez *et al.* 2004). Initially many organisms are responsible for fermentation, but as the alcohol content of the must increases, *Saccharomyces cerevisiae* achieves dominance due to its ethanol tolerance. Rementeria *et al.* (2003), refer to the ecology of spontaneous yeast fermentations of the regionally unique Basque *Txakoli* wine. A common problem in these spontaneous fermentations was that wines became stuck and ceased to ferment. As a result, Rementeria *et al.* (2003) undertook to characterise the microbiota of the wine as a first step in the selection of an autochthonous (indigenous or aboriginal) yeast strain. In terms of traditional beverages, the *Txakoli* wines are at the same evolutionary point as *iQhilika* and many other beverages which cross from traditional

to scientific production systems, and hence from random inoculi to more carefully controlled inocula. The use of controlled inocula results in wine of a generally higher quality (Regodon *et al.* 1997). This does not mean that products of spontaneous fermentations are inferior, but rather that products of inoculated fermentations are more predictable.

3.1.3 Inoculated fermentations

Where expensive substrates such as honey, grape juice or beer wort are being fermented, unpredictable spontaneous fermentations involving excessive spoilage organisms produce products of low quality resulting in financial loss (e.g. Rementeria *et al.* 2003, Sakamoto & Konings 2003). Within the wine industry the development of characteristic wine-making strains has resulted in the production and marketing of these strains on a global level, a phenomenon which has in turn resulted in researchers and producers in specific wine making regions developing yeasts that are specific and characteristic of indigenous favourable spontaneous fermentations (Regodon *et al.* 1997). The use of these strains is believed to be important in that the yeasts are adapted to the specific regional characteristics thus maintaining the sensory characteristics of the spontaneous wines of the region.

Saccharomyces cerevisiae and its many unique strains are responsible for the dominant characteristics of today's beers and wines (e.g. Hough 1984, Pretorius 2000). Other characteristics may be imparted to fermentation products by bacteria such as lactic acid bacteria (Nedovic *et al.* 2000, Alexandre *et al.* 2004) as part of secondary maturation fermentations. With the availability of typical wine-making yeasts and wine maturation bacteria, wine-making has become a dependable source of marketable products leading to the rapid growth in wine production seen globally.

3.1.4 The evolution of fermented beverages

The previous sections have detailed the advances in the predictability of the outcomes of fermentation allowed by the isolation and cultivation of specific micro-organisms for use in specific processes. A search through the literature on yeast

ecology in wine for example shows that the many papers published are funded by either governments of wine making countries or by wine making bodies. The research yields results which increase the quality and competitiveness of the products.

Mead making is largely a traditional industry and the governments of Ethiopia, Zambia, South Africa and Tanzania do not derive tax revenue from the sale of traditional mead. As a result, this industry has not generated revenue for research and development and it has evolved at a slower rate than wine or beer.

3.1.5 Potential sources of microbes in *iQhilika*

3.1.5.1 Honey

Honey is the concentrated nectar of flowers as collected and processed by honeybees (Snowden & Cliver 1996, Devillers *et al.* 2004). Barnett *et al.* (1983) list of over 830 known described yeast species. A large percentage of these were isolated from flowers and many more from honeybees themselves.

The honeybees in a beehive make tens of thousands of forays per day up to a range of 6-10km (Seeley 1995) with foragers visiting up to 100 flowers per foray. This translates to a foraging area between 113 and 314 km² per beehive, with a beehive being exposed to nectar from millions of individual flowers per day. The potential concentration of micro-organisms through foot contact with flowers and comb surfaces is, as a consequence, considerable.

Snowdon & Cliver (1996) mention that in terms of yeast contamination, honey contains on average below 100 colony forming units per gram and can contain spores of at least 25 different genera of bacteria. It is interesting to note however that Gilliam (1997) found very few yeasts associated with bees themselves and when yeasts were present they were more often found in stressed or diseased bees. Many bacterial species are mentioned by the above authors as having been isolated from certain bees or certain honey samples, but it is important to note that the bees are believed to actively encourage the growth of gluconic acid producing bacteria in ripening honey (Ruiz-Argueso & Rodriguez-Navarro 1973). As a consequence it would be expected that these microbes and their spores may be found in honey.

3.1.5.2 Plant roots

Fox & Norwood-Young (1982), list a number of African plant species which are used as yeast sources in fermented beverage making, including *Trichodiadema* sp. which are reported by traditional brewers to be the yeast source for *iQhilika* production. Juritz (1906) suggested that the roots of *Trichodiadema* may contain yeast. Van Wyk & Gericke (2000) listed a number of traditional yeast roots used in southern and central Africa and their corresponding beverages, namely *Avonia ustulata* - Moerplantjie (mead and honey beer), *Avonia papyracea* – Kareemoerplantjie (mead and honey beer), *Khadia acupetala* Khadi Root, Khadiwortel (mead and beer), *Peucedanum sulcatum* Bierwortel, (mead). Mitchell & Breyer-Brandwijk (1962) and Smith *et al.* (1950), list *Trichodiadema stellatum* as a source of yeast for both mead and bread making. It is interesting to note that the Afrikaans word for yeast is 'moer' and for root is 'wortel' and beer is 'bier'. As a result, the Afrikaans names, when translated to English are; little yeast plant; little karee yeast plant; Khadi root and beer root. Tsuyoshi *et al.* (2004), report on the preparation of traditional microbial starter cultures in Asia, where the roots of *Plumbago zeylandica* are utilized, in conjunction with ginger roots – showing an example from outside the African continent of a plant root being used in a yeast preparation.

3.2 Objectives

To determine:

- the identity of the dominant yeast/s in *iQhilika*; and
- if these produce the dominant organoleptic properties of *iQhilika*.

A secondary objective, if it emerged as part of the inquiry into the above factors, was to ascertain the origin of the yeast.

3.3 Materials and methods

In order to determine the identities of the dominant fermentative organisms in *iQhilika*, the following philosophical strategy was used.

iQhilika samples were taken from two places 350km apart. The fermentative organisms from these samples were isolated and re-inoculated into fresh media to prove whether a specific isolate is, or is not, responsible for the dominant features of *iQhilika*. The causative isolates from the different regions would then be compared to ascertain if *iQhilika* is produced by a spontaneous fermentation or an inoculated fermentation with the fermentative organism/s derived from a known wild source.

3.3.1 Sample sites

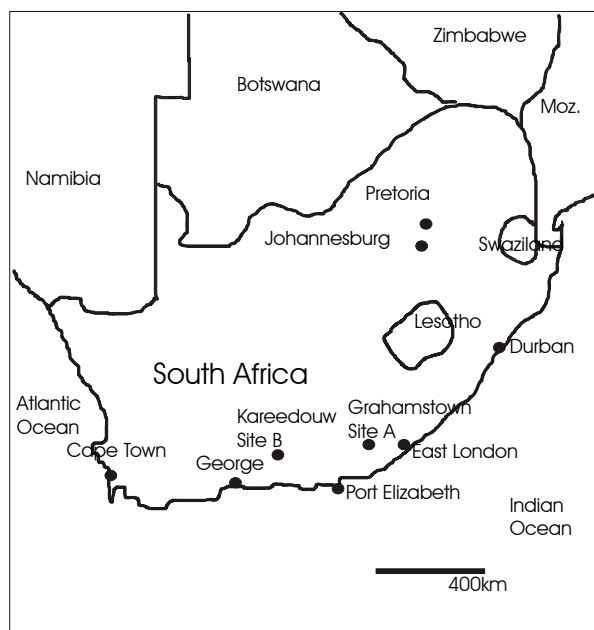


Figure 3. 1 A map of South Africa showing sample site A and sample site B separated by a distance of approximately 350 km. Sample site A is Grahamstown, sample site B is Kareedouw. It is interesting to note that the X'ai (Khoi-San tribe) word for *iQhilika* is Karee (Van Wyk & Gericke, 2000). The samples from Kareedouw were taken during the Karri fees – the modern day festival of Karee.

In this study two sample sites 350 km distant to each other were selected (Figure 3.1). Three samples were taken from site A and 5 samples from site B. More samples were obtained in Kareedouw than Grahamstown due to the larger number of brewers represented in the area. The dominant fermentative micro-organisms in beverages from each sample site were identified and compared to each other to ascertain whether common strains were evident.

3.3.2 Yeast isolation

Samples of *iQhilika* were drawn from batches of *iQhilika* from site A and Site B. A 100 fold serial dilution was performed on all samples and 10µl plated onto malt extract agar (DIFCO) and lysine agar (DIFCO). Malt Extract agar is a general media for the isolation of yeast and fungi (Van der AA Kuhle & Jespersen 1998) and lysine agar specifically selects for wild yeasts (Atlas & Parks 1993). In a preliminary study it was observed that the *iQhilika* yeasts grow on lysine agar.

The Kareedouw yeast isolates were named A, B, D, E, F and Grahamstown isolates W, WG and DG.

3.3.3 Identification of yeast isolates

3.3.3.1 Phenotypic identification

Colonies were grown on wort agar at 28°C for 56 hours and examined microscopically for basic morphology. Specific attention was paid to the development of pseudohyphae as discussed by Gancedo (2001). The ability of cells to flocculate at the end of a fermentation is considered a desirable trait in wine yeast (Shinohara *et al.* 1997, Pretorius 2000). Pseudohyphae formation is triggered by nitrogen limitation and can cause sedimentation, a factor which in traditional mead fermentation systems is seen as undesirable as fermentations may progress too slowly as a result (Steinkraus & Morse 1966). For continuous fermentations, sedimentation and flocculation, as well as adsorption of cells to supports are useful traits which can be employed to retain biomass in a bioreactor. Hence in a continuous system, or a fed batch system, cells which sediment due to pseudohyphal growth will be useful.

3.3.3.2 Physiological identification techniques

Rohm *et al.* (1990) describes the use of API32C (bioMerieux) yeast identification strips to identify a wide range of food borne yeasts. Van der AA Kuhle & Jespersen (1998), report on the use of API32C identification strips in identifying wild yeast isolates from lager breweries.

The API32C tests consist of a number of wells containing various carbon sources, which when inoculated with a pure isolate can indicate the ability of that isolate to grow on the specific carbon source (bioMerieux 1993). The carbon assimilation profiles are then compared to a database of recorded profiles for many different yeast species and a possible identification is made. In addition these tests give a useful carbon assimilation profile for a specific isolate.

3.3.3.3 Molecular identification

Single colonies were selected and grown overnight on an orbital shaker in 100 ml yeast nitrogen basal media (DIFCO) with 5 g/l glucose. Colonies were harvested by centrifugation and DNA was extracted using a Qiagen Tissue DNA extraction kit (yeast protocol).

A PCR product in the region of 600 bp was amplified using the universal primers described by Fell (1993) which are specific for a section of the V3 variable region of the large ribosomal subunit gene. This area is highly conserved within all organisms (Jeeninga 1997). A master mix containing 5 µl (25U) TaKaRa Taq™ DNA polymerase, 100 µl TaKaRa PCR buffer, 60 µl TaKaRa MgCl₂, 80 µl TaKaRa dNTP's 30 µl DNA primers and 300 µl of ultra pure water was prepared.

PCR reactions were performed in a total volume of 50 µl with 10 ng DNA extract as follows: 93°C for 2 minutes, followed by 30 cycles of 93°C for 30 seconds, 66°C for 90 seconds and 72°C for 20 seconds, with a final extension at 72°C for 4 minutes. Reactions were performed on a Hybaid thermal cycler. Aliquots of 2 µl of each sample were electrophoresed on a 1% agarose gel containing ethidium bromide to check for the 600 bp fragment.

The amplified fragments were sequenced in both directions by the Direct Dydeoxy Chain Terminating Sequencing Method on an ALF Express system (Amersham Pharmacia), as per the manufacturers instructions. The sequence data was produced using ALFwin Sequence Analyser 2.00 software.

Sequence data was compared with data available at www.ncbi.nlm.nih.gov (12 April 2000) using the BLAST (Altschul *et al.* 1990) software package as with data

available at the Ribosomal Database Project (Larsen *et al.* 1993, Maidak *et al.* 1996) www.cme.msu.edu/RDP (12 April 2000). Similar sequences for a variety of *Saccharomyces* species were located using the BLAST search and used to perform a multiple sequence alignment together with the sequences from the *iQhilika* isolates. This alignment was used to infer 13 un-rooted parsimonious phylogenetic trees using Paup V4.0. These were used to generate a consensus tree and a neighbour joining tree using the same software.

James *et al.* (1997) analysed 18S rRNA gene sequences of the genus *Saccharomyces* and using this data were able to separate *Saccharomyces* species into three clusters. A similar strategy as was used in this study to ascertain where the *iQhilika* isolates could be placed within these groupings.

3.3.4 Fermentation characteristics

In terms of beer yeast fermentation characteristics, Hough *et al.* (1971) recognize six types of behaviour:

Group A: yeasts that sediment early in the fermentation because of flocculation, but fermentation proceeds because of continued action of the sedimented yeast.

Group B: yeasts that only sediment when the wort is well attenuated and simultaneously fermentation is substantially arrested.

Group C: yeasts that sediment only to a small degree at the end of the fermentation, leaving much of the yeast in suspension.

Group D: yeasts that pass out of suspension into a yeast-head early in fermentation and fermentation is prematurely arrested.

Group E: yeasts that form a yeast-head to some extent, but much is left in suspension. When the wort is well attenuated, the suspended yeast sediments.

Group F: yeasts that form a yeast-head to some extent but the greater part of the yeast remains suspended and does not sediment.

To assess basic fermentation characteristics of the isolates, these fermentation properties were assessed.

Colonies of each strain were inoculated into 1 litre each of growth medium [40 g/l wort extract (Difco), 20 g/l glucose (Merck)] and left on a shaker at 25°C for two

days. Cells were harvested by placing them at 4°C for one day so as to allow them to settle. The spent medium was poured off and the yeast was placed in 2 litres of sterile autoclaved honey solution with a specific gravity of 1.055. Fermentation was allowed to continue for 5 days during which time the nature of the yeast was observed and its fermentation group ascertained.

The flasks were then placed at 4°C for two days to allow the yeast to settle out completely. The resulting beverage was drawn. Samples were tasted by traditional brewers and the similarity, in terms of taste and bouquet, to traditional *iQhilika* was assessed. If an isolate produced a product which tasted similar to the original and was judged by traditional brewers to be desirable, the isolate was likely an *iQhilika* producing strain.

In preliminary research it was found that the cultivation of yeast cells in microtitre plates provided meaningful growth data until such a point that the yeast cells flocculate, after which the data is distorted. This does however provide a simple method for determining the flocculation characteristics of the yeast isolates.

Honey varies greatly in composition, but consists predominantly of the two sugars glucose and fructose (Snowden & Cliver 1996, Terrab et al. 2004). Hence an artificial honey solution consisting of 75 g/l glucose (Merck) and 75 g/l fructose (Merck) was prepared. To aid fermentation the following salts were added: 7 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 32 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 32 mg/l KH_2PO_4 , 128 mg/l $(\text{NH}_4)_2\text{SO}_4$.

Solutions containing the above ingredients were adjusted to pH 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5 and 6 using 2 M tartaric (Merck) and 2 M KOH (Alpha) solutions.

The growth medium was filter sterilized (Cameo 0.22 µm cellulose acetate syringe filter) and 100 µl was auto-pipetted into each well of a 96 well microtitre plate (Merck). All wells were inoculated with log phase (OD_{600} of 0.15) yeast of one of the strains being tested (A, B, D, E, F, W, WG, DG). Light mineral oil (10 µl) was placed over all wells to limit evaporation and allow semi anaerobic conditions to prevail in the well.

The micro titre plate was placed in a Bio-Tek Instruments Powerwave X micro-titre plate reader. The Powerwave X instrument was controlled using K-C Junior V 1.31.2 Software. The reader was set to incubate the plate at 25°C. Readings were taken at 600 nm every 30 minutes for 43.5 hours.

3.3.5 Enzyme profiles of isolates

The API Zym (bioMerieux) is a 20 well strip that tests for the presence of certain enzymatic reactions.

Colonies of each strain were used to inoculate one litre of yeast malt extract broth (DIFCO) and left on a shaker at 25°C for two days. Cells were harvested by placing them at 4°C for one day to allow them to settle. A 0.5 ml aliquot of cells was withdrawn from the sediment and resuspended in fresh medium.

The API Zym tray wells were inoculated with 65 µl of the suspensions and incubated at 28°C for 4 hours. One drop of Zym A and one drop of Zym B reagents supplied with the API Zym Kit were placed in each well and the strips were left for 5 minutes, after which they were placed under a 1000 watt light bulb for 10 seconds as per the manufacturer's instructions.

The strips were scored as per the instructions for enzyme activity and the results recorded on the supplied API data sheets.

3.3.6 Electron microscopy

Samples were prepared as in Chapter 2, section 2.3.1.4.

3.4 Results

3.4.1 Phenotypic identification

Preliminary microscopic examination showed that all 8 isolates were single celled budding yeasts.

Moderate pseudo-hyphal growth was seen in isolates A, D, F and W. Pseudohyphae consisted of short strings of cells with no elongation of individual cells.

3.4.2 Physiological identification

The identification of isolates using the API 32 C yeast identification strips showed that all isolates had carbohydrate assimilation profiles which were similar to those for known strains of *Saccharomyces cerevisiae* (Table 3.1).

Table 3. 1 API 32C carbon assimilation profiles for yeast isolated from *iQhilika* samples.
Symbols: N = no utilisation, Y = utilisation. SC is the API32C code for *Saccharomyces cerevisiae*.

Substrate	Isolate							
	A	B	D	E	F	W	WG	DG
Pentoses								
L-Arabinose	N	N	N	N	N	N	N	N
D-Xylose	N	Y	N	N	Y	N	N	N
Ribose	N	Y	N	Y	Y	Y	N	N
Hexoses								
Methyl-D-glucoside	Y	N	N	N	N	N	Y	N
Galactose	Y	Y	Y	Y	Y	Y	Y	Y
Glucose	Y	Y	Y	Y	Y	Y	Y	Y
Sorbose	N	N	N	N	N	N	N	N
Rhamnose	N	N	N	N	N	N	N	N
Disaccharides								
Cellobiose	N	N	N	N	N	N	N	N
Maltose	Y	N	Y	Y	Y	Y	Y	Y
Lactose	N	N	N	N	N	N	N	N
Melibiose	N	N	N	N	N	N	N	N
Sucrose	Y	Y	Y	Y	Y	Y	Y	Y
Trehalose	Y	Y	Y	Y	Y	Y	Y	Y
Palatinose	N	N	Y	N	Y	N	N	N
Trisaccharides								
Melezitose	N	N	N	Y	Y	Y	N	N
Rafinose	Y	Y	Y	Y	Y	Y	Y	Y
Alcohols								
Glycerol	N	N	N	Y	N	N	N	N
Erythritol	N	N	N	Y	Y	N	N	Y
Mannitol	N	N	N	N	N	N	N	N
Inositol	N	N	N	N	N	N	N	N
Sorbitol	N	Y	N	N	N	N	N	N
Organic Acids								
Glucuronate	N	N	N	N	N	N	N	Y
DL-Lactate	Y	N	Y	Y	Y	N	Y	N
2-Keto-gluconate	N	N	N	N	N	N	N	N
Levulinate	N	N	N	N	N	N	N	N
Gluconate	N	N	N	N	N	N	N	N
Amino Acids								
N-Acetylglucosamine	N	N	N	N	N	N	N	N
Glucosamine	N	N	N	N	N	N	N	N
API32C Identification								
API32C Identification	SC	SC	SC	SC	SC	SC	SC	SC

3.4.3 Molecular identification

The sequences obtained (Figure 3.2) were aligned to *S.cerevisiae* (YSCRGIH5).

S.cerevisiae	CGTTCCTTGCTATGTTCTTGGAAACAGGACGTCATAGAGGGTGAGAATCCCGTGTG
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	GCGAGGAGTGCGGTTCTTTGTAAAGTGCCCTTCGAAGAGTCGAGTTGTTTGGGAATGC
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	AGCTCTAAGTGGGTGGTAAATTCATCTAAAGCTAAATATTGGCGAGAGACCGATAG
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____C_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	CGAACAAGTACAGTGATGGAAAGATGAAAAGAACTTTGAAAAGAGAGTGAAAAAGTA
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	CGTGAAATTGTTGAAAGGGAAGGGCATTGATCAGACATGGTGTGTTTGTGCCCTCTG
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	CTCCTTGTTGGGTAGGGGAATCTCGCATTTCACTGGG CCAGCATCAGTTTGTGGTGGC
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____G_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

S.cerevisiae	AGGATAATCCATAGGAATGTAGCTTGCCTCGGTAAGTATTATAGCCTGTGGGA T
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____G_A_____
Isolate DG	_____G_A_____

S.cerevisiae	ACTGCCAGCTGGGACT GAGGACTGCGACGTAAGTCAAGG ATGCTGG CATAATGG
Isolate A	_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____T_____G_____
Isolate W	_____
Isolate WG	_____A_G_____C_G_____
Isolate DG	_____C_____

S.cerevisiae	TTATATGCCGC
Isolate A	G_____
Isolate B	_____
Isolate D	_____
Isolate E	_____
Isolate F	_____
Isolate W	_____
Isolate WG	_____
Isolate DG	_____

Figure 3. 2 Comparison of the partial sequences of the V3 rRNA gene sequences for *Saccharomyces cerevisiae* (YSCRGIH5) and the eight strains isolated. Where differences were found they are indicated by the base, or lack thereof as a space.

The sequencing of the V3 rRNA gene sequences showed a number of variations from the *S.cerevisiae* (YSCRGIH5) (Figure 3.2). When used to infer a phylogenetic tree the isolates formed a group (Figure 3.3).

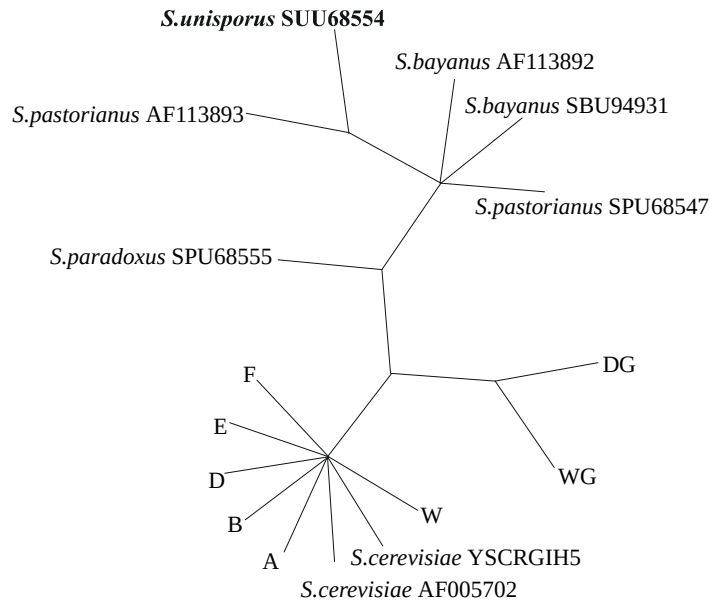


Figure 3. 3 A consensus tree derived from 14 un-rooted parsimonious trees inferred using PAUP V4.0 from the alignment of the partial sequences of the V3 rRNA with the sequences obtained for known *Saccharomyces species* (Genbank).

The data was then used to compile a neighbour joining tree (Figure 3.4).

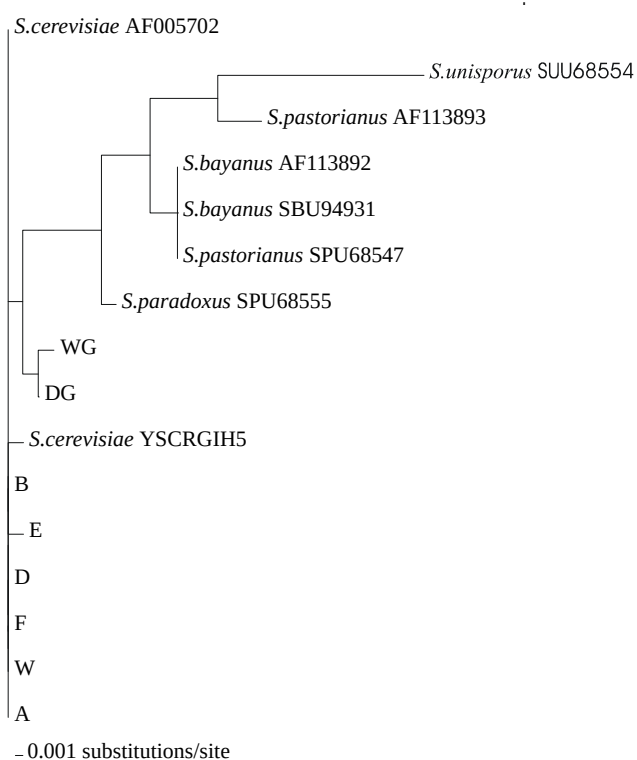


Figure 3. 4 A neighbour joining tree compiled using PAUP V4.0 for the same data set used in Figure 3.3.

3.4.4 Yeast enzymes

The API Zym kit detected the following enzymatic activities (Table 3.2) for the isolates. BioMerieux, the manufacturers of the kit, emphasise that the API Zym kit does not necessarily detect the presence of a specific enzyme, but rather the ability of a specific enzyme to break down a substrate that is broken down by a known enzyme.

Table 3. 2 API Zym Enzyme profiles obtained. It is important to note that a colour reaction in a well does not necessary mean that the enzyme specified by the API Zym kit is present, but rather that an enzyme capable of degrading the same substrate is present. Y= reaction, N = no reaction.

	Isolate							
Enzyme	A	B	C	D	F	W	WG	DG
Alkaline Phosphatase	Y	Y	Y	Y	Y	Y	Y	Y
Esterase (C4)	N	Y	Y	Y	Y	Y	Y	Y
Lipase (C14)	N	N	N	N	N	N	N	N
Leucine arylamidase	Y	Y	Y	Y	Y	Y	Y	Y
Chymotrypsin	N	N	N	N	Y	Y	Y	Y
Acid Phosphatase	N	Y	Y	Y	Y	Y	Y	Y
Napthol-AS-BI-phosphohydrolase	Y	Y	Y	Y	Y	Y	Y	Y
α -glucosidase	N	N	N	N	Y	N	N	N
β -glucosidase	Y	N	N	N	N	N	N	N

3.4.5 Electron microscopy

SEM analysis of both root sections and yeast isolates showed certain important aspects of the growth of yeast in the *imoela* biomass preparation.

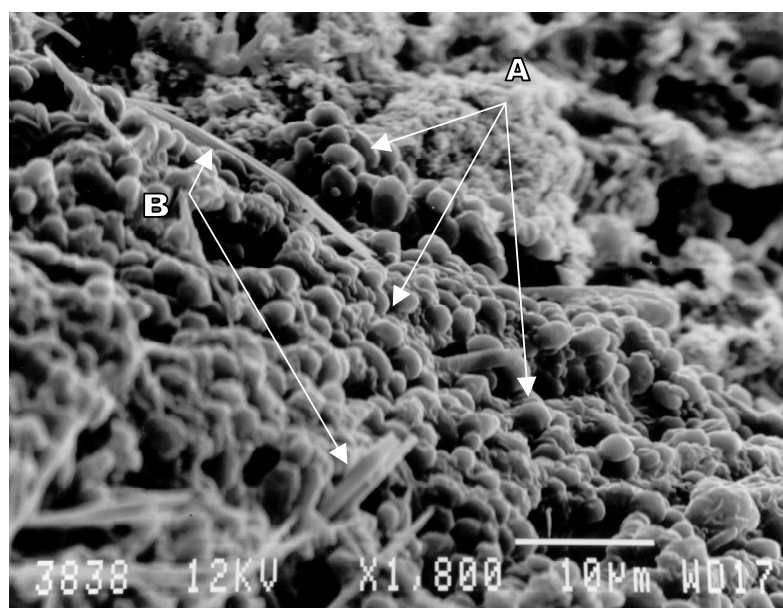


Figure 3. 5 A scanning electron micrograph (SEM) of a section of *Trichodiadema intonsum* immobilised biomass. A large number of yeast cells (A) are evident, as well as insoluble organic crystals (B).

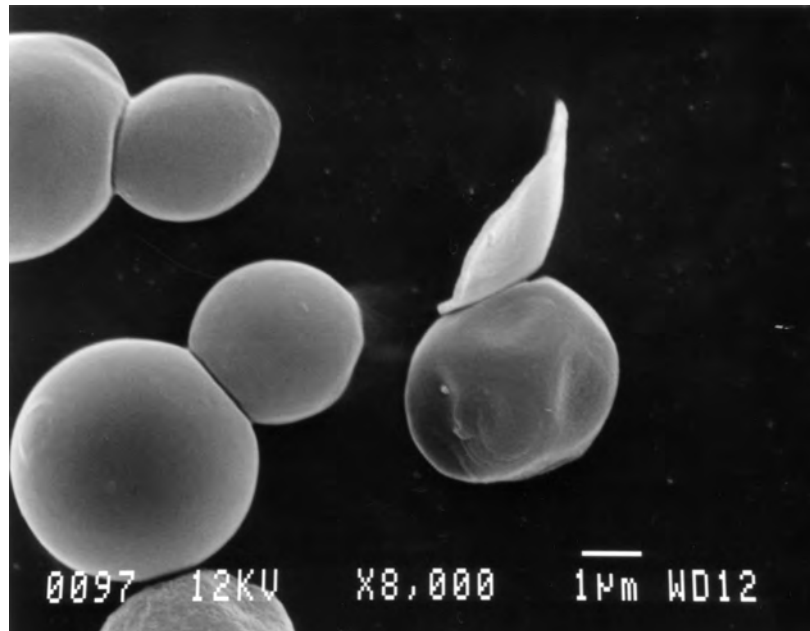


Figure 3. 6 An SEM of a typical example of strain F drawn from a fresh colony on an agar plate. It is evident from this micrograph that the yeast is a budding yeast.

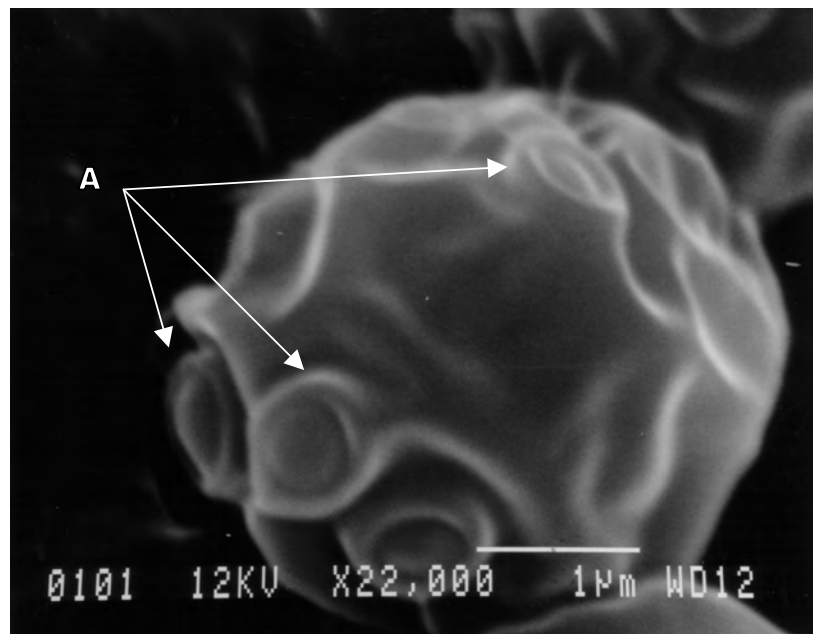


Figure 3. 7 An SEM of an older yeast cell of strain F isolated from a batch of mead. Note the large number of budding scars (A) on the cell.

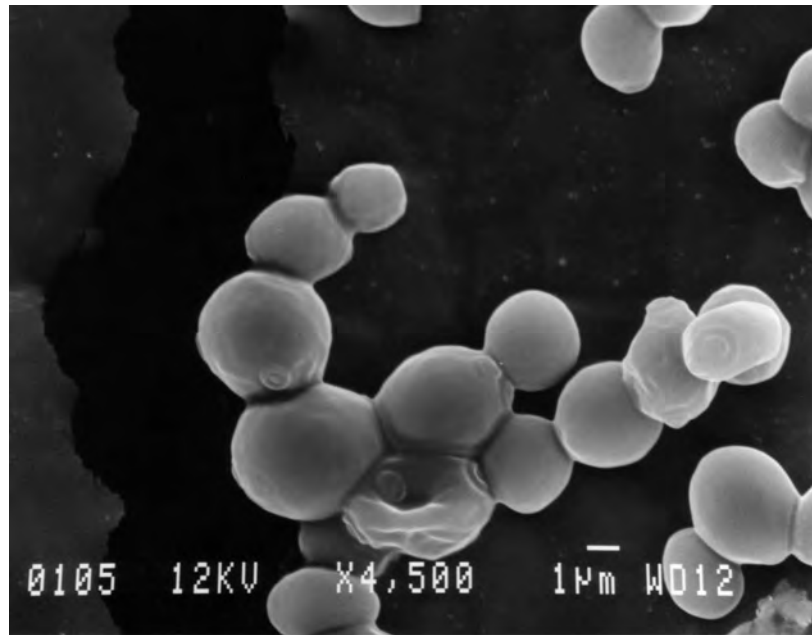


Figure 3. 8 On nutrient limiting media containing honey only, cells of strain F formed strings, possibly pseudohyphae, and became highly flocculent. In this SEM a number of yeast cells are visible which appear to have all budded from one mother cell and did not subsequently become independent free cells.

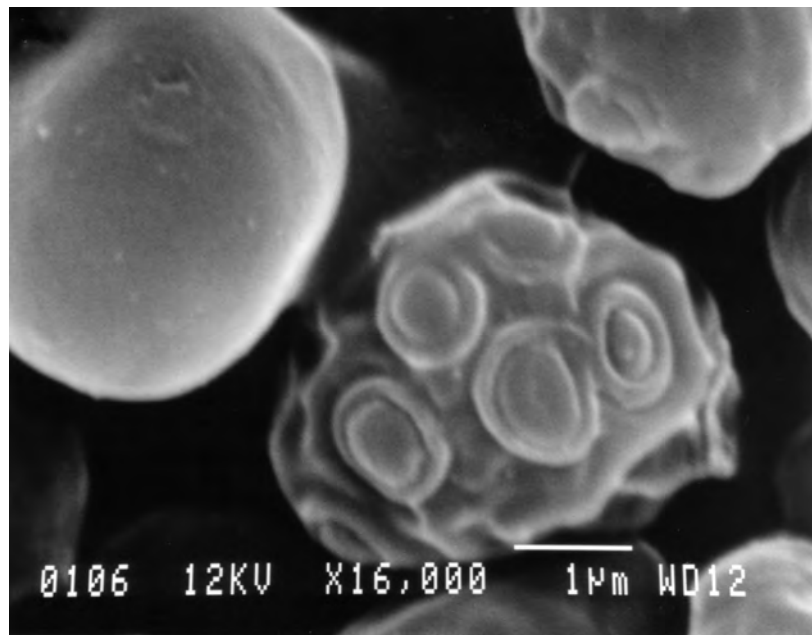


Figure 3. 9 The central cell in this SEM appears to have lysed. Within the imoela biomass preparation a large number of older cells such as this are evident. Some retain a spheroid shape, while others implode. This may be a result of the critical point drying process, or may be that the cells had lysed. Given that many cells with this many scars had not imploded, it is likely that the cells were in fact lysed naturally.

3.4.6 Organoleptic analysis by traditional brewers

The independent organoleptic analyses by Mr Gadu and Mr Makwana yielded very similar results (Table 3.3).

Table 3. 3 Outcomes of organoleptic assessments of beverages produced with various isolates.

Brewer	Isolate							
	A	B	D	E	F	DG	WG	W
Mr D. Gadu	Very Weak	Strange	Acceptable	Very Weak	Excellent	Acceptable	Strange	Excellent
Mr M. Makwana	Very Weak	Woody	Acceptable	Very Weak	Excellent	Acceptable	Strange	Excellent

3.4.7 Fermentation characteristics

Flask fermentations to assess the various isolates against the classification scheme of Hough *et al.* (1971) provided the results in Table 3.4.

Table 3. 4 The fermentation characteristics of the isolates in batch culture classified according to the system of Hough *et al.* (1971) listed in section 3.3.4 of this chapter.

	Isolate							
	A	B	D	E	F	DG	WG	W
Fermentation type	A	C	C	A	B	C	C	B
Description	Weak fermenter	Rapid fermenter	Rapid fermenter	Weak fermenter	Rapid fermenter	Rapid fermenter	Rapid fermenter	Rapid fermenter

In Figure 3.9 the onset of flocculation is visible at different pH values as the point at which optical density of the culture rapidly dropped.

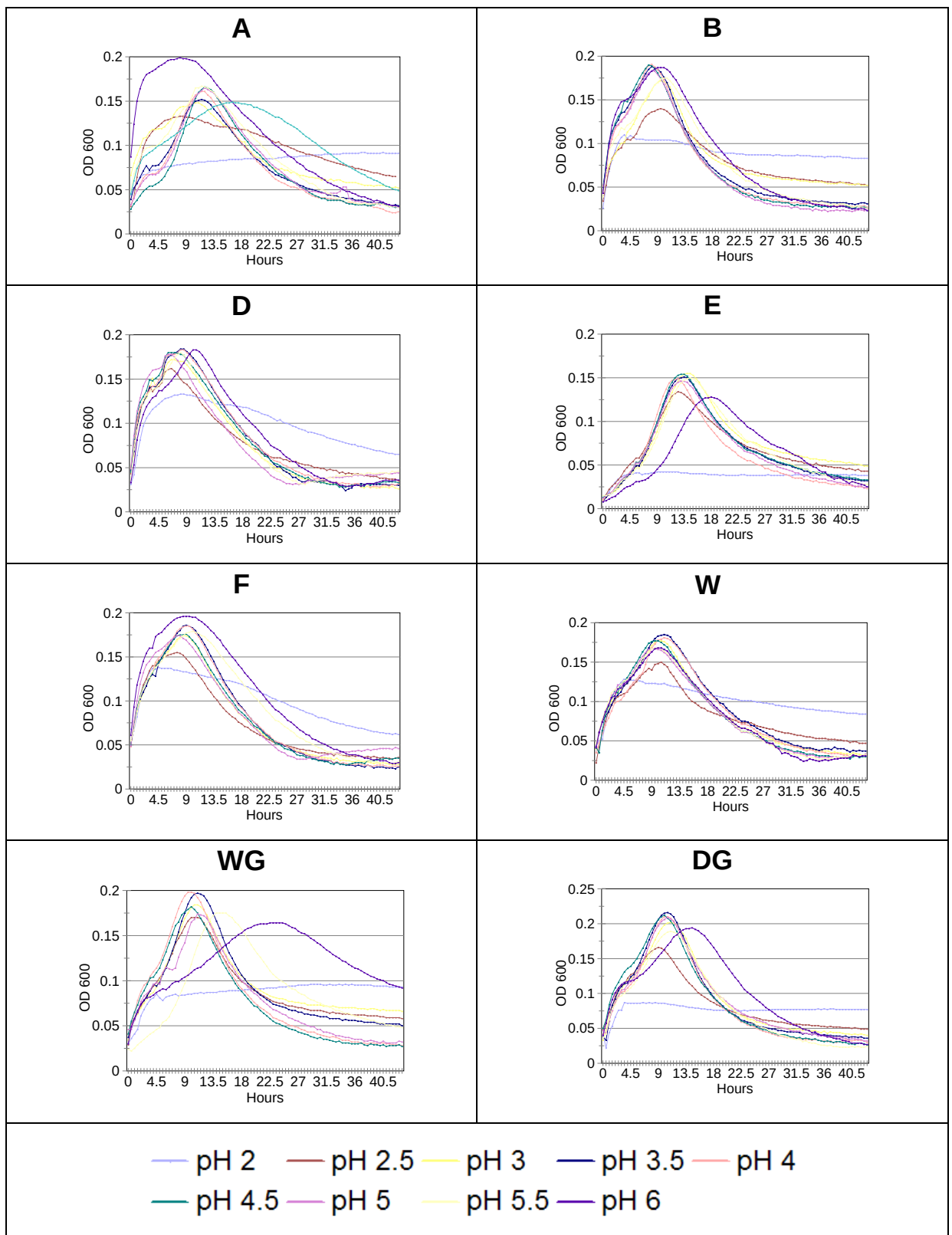


Figure 3.10 The growth and flocculation of the various strains at different pH values is evident as an increase in optical density followed by a rapid drop in optical density at the onset of flocculation.

3.5 Discussion

The eight isolates all fulfilled the criterion of producing a beverage similar to the original. Traditional brewers rated isolates F and W as producing the most desirable fermented product and A and E as producing the least desirable products on account of their weakness and lack of fermentation.

In terms of physiological factors, the isolates were essentially similar in terms of their identification by the API32C kit, being identified as *Saccharomyces cerevisiae*, with certain unusual fermentation characteristics, such as the ability of strains B and F to grow weakly for one or two cell divisions on Xylose (Table 3.1). All the isolates were budding yeasts.

Scanning electron micrographs of the imoela (*Trichodiadema intonsum* immobilised biomass) showed that cells appeared to be entrapped in a mucin matrix (Figure 3.4) similar to that shown by Vadasz *et al.* (2000). This matrix is likely to assist in maintaining a biofilm on the root supports. Crystals of some sort are evident in the imoela (Figure 3.4). Burt-Davey (1912), in an analysis of the closely related *Mesembryanthemum mahoni*, noted the presence of considerable amounts of insoluble oxalate in the roots of *M.mahoni*, but the crystals in figure 3.4 do not have the shape of oxalate crystals.

The isolates from Grahamstown and Kareedouw formed a mixed group based on genetic analysis, with no particular order of relatedness between the yeasts within one sample site, but a close relationship between the yeasts from both sites (Figure 3.3). The isolates identified by traditional brewers as being the most desirable, namely W and F, from Grahamstown and Kareedouw respectively, were very closely related, and exhibited remarkably similar fermentation characteristics (Table 3.4). The beverages produced with these strains were judged by traditional *iQhilika* producers to have the most desirable properties (Table 3.3). The two less desirable Grahamstown strains, WG and DG formed a small branch off the main trunk between *S.cerevisiae* and the other *Saccharomyces* species. The majority of strains of *iQhilika* yeast are however within the *S.cerevisiae* cluster.

James *et al.* (1997) analysed 18S rRNA gene sequences of the genus *Saccharomyces*. They note that although the genus was phylogenetically very heterogeneous with members of other genera being interspersed, there were three distinct clusters of *Saccharomyces* species. The first cluster included four species of the *Saccharomyces sensu stricto* complex, *S.bayanus*, *S.cerevisiae*, *S.paradoxus* and *S.pastorianus*. The second such cluster consisted of *S.castelli* and *S.dairensis*. The third cluster contained *S.servazzii*, *S.unisporus* and a strain CBS 6904. *S.exiguus* is reported to have formed a close group with *Candida holmii* and *S.barnettii*.

The isolates from *iQhilika* fell within the *Saccharomyces sensu stricto* complex to form a closely related cluster of African brewing yeasts (Figure 3.3). The form of this cluster is similar to that of the *S.pastorianus/bayanus* branch of the phylogenetic tree compiled by James *et al.* (1997). This cluster and the *iQhilika* cluster are both beverage associated groups.

The brewing characteristics within the *iQhilika* cluster were however considerably divergent, with some yeasts exhibiting characteristics which were not completely desirable in terms of the continued brewing of *iQhilika* (Tables 3.3 & 3.4). Isolates F and W formed a good suspension for the first portion of the fermentation process, whereupon they settled into a yeast bed, with a reasonable amount still in suspension. These isolates were however judged as the best isolates, and were also isolated from beverages where the brewers had been using their iMoela/moer preparation for in excess of one year. The other isolates from Kareedouw were from younger beverages. Chen *et al.* (1994) reported on the rapid adjustment of a flocculent *Saccharomyces cerevisiae* strain to a system with a high cell washout rate. The ability to flocculate or form pseudohyphae in wine yeasts is reportedly induced by certain structural flocculation genes (Lo & Dranginis 1998, Vadasz *et al.* 2000). These genes govern among other things the secretion of mucin, which would aid adhesion to the *Trichodiadema intonsum* roots. Repeated removal of media from the *bioreactor* and addition of fresh must would lead to a rapid selection for yeasts that could flocculate and adhere to the supports and thus not be consumed with the beverage. In this regard, it is possible that the selective pressures imposed by the repeated use of an *iQhilika* yeast preparation lead to the selection of strains of the

iQhilika Saccharomyces cerevisiae brewing cluster which have the fermentation characteristics of strains F and W, and hence the genotype of F and W.

Scanning electron microscopy of flocculated Strain F yeasts showed that the yeast cells had strung together into 'necklace like' structures (Figure 3.7), which were similar to the pseudohyphae described by Vadasz *et al.* (2000) and Gancedo (2001), consisting of aggregated strings of cells which have a more rapid sedimentation rate than individual cells. Cells from freshly growing cultures appeared to be single celled budding yeasts (Figure 3.5).

The flocculation of the yeast cells appears to be a time related factor with all strains flocculating after 9-13 hours (Figure 3.9). Flocculation is delayed at higher pH values. In Figure 2.14 in Chapter 2 a gradual decrease in pH from pH 4.5 at the initiation of fermentation to pH 3.7 at the end of fermentation was observed. With certain honey types characterised by a pH ranging from 6-7, the increase in pH as fermentation progresses may be desirable to ensure flocculation of biomass and hence retention of fermentative capacity between batches.

In the making of certain traditional beverages, autolysis of yeasts contributes a range of flavours such as those contributed in champagne and sparkling wines (Gonzalez *et al.* 2003). The flavours of traditional *iQhilika* have a certain earthy yeasty flavour which could quite likely be a product of yeast autolysis. Alkaline phosphatase is typically an intracellular enzyme, hence its detection suggests cell lysis (Table 3.2). Strains F and W showed the strongest alkaline phosphatase activity and were also the strains preferred by traditional brewers. Microscopic analysis of yeast at various points during the fermentation cycle showed no major increase or decrease in cell viability, but at all times a small number of lysed cells were visible. Scanning electron microscopy of the cells showed that older cells characterised by many bud scars (Figure 3.6) often appeared to have lysed (Figure 3.8). It is likely that autolysis of yeast cells in *iQhilika* is an important part of the production process as far as flavour is concerned. This is similar in many ways to sparkling wines (Pretorius 2000, Martinez-Rodriguez *et al.* 2001, Gonzalez *et al.* 2003) and sherry (Charpentier *et al.* 2004) where autolysis of yeast contributes organoleptic qualities unique to the product.

The source of the yeast was not determined, but the consistency of yeast used in *iQhilika* making was. All isolates were genetically similar and an enrichment process appears to select for the yeast with the desired characteristics allowing a dominance of desired fermentative organisms to be maintained. It would be interesting to pursue the actual source of the yeast, but this is outside the scope of this thesis.

Crane (1999) suggests that from circa 1300 AD prepared yeast was used as an adjunct to increase fermentation and increased the alcohol content of the beverage. Figure 1.7 in Chapter 1 shows a map of Africa with the distribution of the Khoi-San 10 000 years ago and now, as well as the distribution of mead making in Africa.

In Chapter 1 it was tentatively hypothesised that the use of adjuncts to initiate fermentation was a traditional technology first employed by the Khoi-San. In Chapter 2 the transfer of fibrous plant materials between batches of mead was shown to retain fermentative biomass.

It is possible that the use of fibrous plant materials to immobilise and enrich for desirable fermentative organisms is a technique dating back 10 000 or more years to the days when the Khoi-San roamed a third of Africa's surface unchallenged by other peoples. It is however safe to state that for considerably longer than 700 years, mankind has, in Africa, been using an enrichment process to prepare a consistent yeast preparation for mead making.

3.6 Conclusion

The objectives of this chapter were to characterise the dominant mycoflora of the *iQhilika* production system. This has been achieved showing that *iQhilika* is a unique African mead produced through the fermentation of honey by a family of strains of *Saccharomyces cerevisiae* which are responsible for its dominant organoleptic characteristics. Some aspect of the brewing process leads to these yeast strains establishing dominance, as yeasts of this family were the dominant micro-organisms found in two regions separated by 350km. Although a scientific strain isolation system is not used to produce an inoculum for *iQhilika* making, it appears that a traditional enrichment method has been developed which has very similar results, selecting for a fermentation consortia of a high level of relatedness. *Saccharomyces*

cerevisiae is a generally regarded as safe (GRAS) microorganism due to its widespread use in beverage production and baking.

According to the South African Liquor Products Act 27 of 1989 discussed in Chapter 1, *iQhilika* can be legally considered as mead under South African law as it uses a *Saccharomyces cerevisiae* strain of yeast, honey and water to produce an alcoholic beverage. The use of brood in *iQhilika* making may not be legal, but the addition of pollen is not illegal as pollen is normally found in honey.

Chapter 4

Lactic acid bacterial populations in traditional iQhilika

4.1 Introduction

In Chapter 3 it was shown that the dominant organoleptic properties of *iQhilika*, as well as the primary fermentation were attributable to, and carried out by *Saccharomyces* yeasts of a close degree of relatedness. Discussions with traditional brewers revealed that a common problem in the yeast preparation used (*imoela*), consisting of biomass immobilised on roots of *Trichodiadema* *sp.* plants, was that with repeated use in producing batches the *iQhilika* produced became weaker in strength and had a more sour taste (Chapter 2). This suggests spoilage.

In Nigeria traditional beverages have very short shelf lives and rapidly undergo deterioration within 48 hours of production (Sanni *et al.* 1999). Traditional African beverages differ from their Western counterparts in being sour, less carbonated and without hops, and are consumed while still actively fermenting (Mwesigye & Okurut 1995). This is in a large part due to the short shelf life of the products. Many consumers of traditional beverages request products that are near the end of their shelf life as the harsh taste of these products is equated with greater strength (Sanni *et al.* 1999). A similar phenomenon has been noted amongst traditional *iQhilika* producers in Grahamstown, where regular *iQhilika* drinkers prefer stronger products (Mr D. Gadu pers. com.).

Sanni *et al.* (1999) detected a number of *Acetobacter* species, certain potential spoilage Lactic acid bacteria (LAB), and wild yeasts in traditional beverages. Both *Acetobacter* and LAB can contribute to souring in beverages by the production of organic acids (Hough 1984). The growth of LAB is not always entirely negative, as in many cases their metabolic products may inhibit the growth of far more pathogenic microbes in the traditional beverage (Tadesse *et al.* 2005). It is however important to note that not all LAB are benign, some may be pathogenic or produce dangerous metabolites (Schleifer *et al.* 1995).

The intentional cultivation of LAB in foodstuffs is often due to the ability of these microbes to produce metabolites which ensure product safety and extend shelf life (Vaughan *et al.* 1994). Beverages such as *Kodo ko jaanr* produced in the Himalayas (Thapa & Tamang 2004), *Boza* in Turkey (Zorba *et al.* 2003), *Kule naoto* in Kenya (Mathara *et al.* 2004), *Pulque* in Mexico (Escalante *et al.* 2004), *Hardiliye* in Turkey (Arici & Coskun 2001), *Borde* in Ethiopia (Tadesse *et al.* 2005), *Kenkey* in Ghana (Olsen *et al.* 1995), *Pozol* in Mexico (Wacher *et al.* 2000), and *Sapal* in Papua New Guinea (Gubag *et al.* 1996) all make specific use of the metabolites of LAB to provide certain flavours and a measure of product safety. The ancient scholars, Herodotus, Pliny and Strabo all refer to the health giving aspects of the millet and barley beverages of Abyssinia (now Ethiopia), Nubia and Egypt (Gayre 1948). Many other foods are also preserved or rendered safer by their fermentation with LAB, such as traditional Greek fermented sausage (Drosinos *et al.* 2005), food condiments in Asia (Leisner *et al.* 1999), and products such as commercial yoghurts and fermented vegetables (Delley *et al.* 1990).

In wine fermentations (Maicas *et al.* 1999), as well as cider (Nedovic *et al.* 2000), the secondary fermentations carried out by the LAB *Leuconostoc oenos*, which has now been renamed *Oenococcus oeni* (Viti *et al.* 1996), are important for the development of the bouquet, subtlety and body of the products.

In order to understand the importance of LAB in contributing both beneficial and non-beneficial characteristics to traditional African beverages, it is useful to briefly review certain of these beverages.

4.1.1 LAB in African traditional beverages other than mead

A larger body of literature exists for traditional African beverages other than mead and in some cases the beverages have been commercialised. Researchers have specifically focused on spoilage of traditional beverages as part of the commercialisation effort.

4.1.1.1 Indigenous beers in Africa

In Africa many different alcoholic beverages are made from grains

Finger millet beverages and millet beverages

Finger millet is made into a mildly alcoholic beverage, with a certain amount of souring contributed by lactic acid bacteria (Mwesigye & Okurut 1995, Gadaga *et al.* 1999). Sugars for fermentation are derived from the malted grains of bulrush millet (*Penisetum typhoideum*) or finger millet (*Eleusine coracana*) in Zimbabwe (Gadaga *et al.* 1999).

The Zimbabwean mildly alcoholic, sweet sour beverage, *mangisi*, is produced by fermenting malted finger millet mixed with flour with the inoculum believed to come from the brewing utensils (Gadaga *et al.* 1999). The characteristic flavour of the beverage is produced by the actions of aerobic mesophilic bacteria, yeasts, moulds and lactic acid bacteria (Gadaga *et al.* 1999).

The Zimbabwean millet beverage, *doro*, is used in important cultural ceremonies, such as the reward of labour parties in agricultural activities, communication with the ancestors, weddings and various other ceremonies (Gadaga *et al.* 1999). Gadaga *et al.* (1999) describe the following recipe for *doro*. The first step involves preparation of the grains in the same way as the *mangisi* described in the previous paragraph, with the product being left to sour in clay pots for approximately two days. The soured product is then boiled to reduce its volume by a quarter after which it stands overnight and the next day about half the volume of malt used in the initial brewing process is added. On the sixth day two to three times the volume of starter material produced in the first day is produced and cooled. A portion of the boiled fermented product (produced on the first day which had had malt added) is strained and put aside. The strainings, the unfermented larger volume, and the remainder of the fermented volume are all mixed and allowed to ferment for two hours. The strained section which was placed aside is then mixed in and the product allowed to ferment until the following day when it is consumed. The product spoils rapidly if stored. The beverage is reported to contain up to 4% alcohol and to be a healthy product due to its vitamin and nutrient content (Gadaga *et al.* 1999).

In Uganda *ajon* is produced from finger millet (Mwesigye & Okurut 1995). The process is essentially similar to that for producing the Zimbabwean *doro*. The beverage is reported to be produced for commercial gain as well as cultural reasons. Again commercialisation of *ajon* is limited by spoilage.

These beverages are similar in many ways to the Asian finger millet beverages of the Himalayas (Thapa & Tamang 2004) in that a certain percentage of the malted millet sugars are converted to alcohols by yeasts and bacteria, and a certain percentage to organic acids such as lactic acid. The beverages are characterised by short shelf lives and are consumed live and unfiltered.

Sorghum beverages

Sorghum beverages in Africa share much in common with the finger millet beverages and are produced and consumed in much the same way (Mwesigye & Okurut 1995, Gadaga *et al.* 1999). During colonial times the beverages were perceived as being healthy and were commercialised for provision to migrant labourers (Gadaga *et al.* 1999). The industrially produced sorghum beer is produced by traditional spontaneous lactic fermentation and alcoholic fermentation with inoculated *Saccharomyces cerevisiae*. In Uganda, sorghum beer is consumed at marriages and social gatherings (Mwesigye & Okurut 1995).

In Xhosa culture, traditional functions are important activities. An uninvited guest will never be turned away, and as such, in poor areas, the consumption of sorghum beer forms a social security net.

Maize beverages

Zea mays, a grass of South American origin was introduced to Africa in the past few hundred years (Mehring *et al.* 1979). Beverages made from maize are adaptations of sorghum and millet recipes and share much in common. Beverages are made from a malted porridge that is often mixed with other grains or consumed on its own (Mwesigye & Okurut 1995, Gadaga *et al.* 1999).

In Uganda, *kweete* is made from maize flour and germinated finger millet (Mwesigye & Okurut 1995), with the maize flour being placed in a pit for a week after which it is

sun dried. The germinated millet is sun dried and ground whereupon it is mixed with water and maize flour and fermented. A stronger version of *kweete* is reported whereby the *kweete* beer is fermented and then water and brown sugar is added followed by fermentation overnight (Mwesigye & Okurut 1995). Production of this beverage is also limited by spoilage bacteria.

4.1.1.2 LAB in the indigenous wines of Africa

Wine in the western world is generally viewed as the beverage produced by the fermentation of the grapes of *Vitis vinifera* (Thudichum & Dupre 1872). Wines are produced in Africa from a variety of fruits. In central Africa wine is made from the juice of special cultivars of banana, producing, *tonto*, with 6-11% alcohol, which due to bacterial spoilage has to be consumed rapidly after production (Mwesigye & Okurut 1995). The Marula tree, *Sclerocarya caffra*, or *Sclerocarya birrea*, subs *caffra* (Venter & Venter 2002) is used to make wine which spoils rapidly due to LAB (Gadaga *et al.* 1999).

In Zambia a wine made from the fruits of *Uapacia kirkiana* (*Masuku*) has been commercialised (Njovu 1993). This commercialisation step has produced a product with far longer storage due to the partial elimination of spoilage bacteria.

4.1.2 Lactic acid bacterial fermentations

LAB are divided into heterofermentative LAB which, when grown in rich nutrient solutions ferment glucose to lactic acid, carbon dioxide, both ethanol and acetic acid, or either one, whereas homofermentative LAB produce only lactic acid (Sneath *et al.* 1986). LAB produce acid both under anaerobic and aerobic conditions (Salminen & von Wright 1998). A specific LAB will produce lactic acid isomers in a specific ratio of + and – lactate (Sneath *et al.* 1986). A defining feature of LAB is their inability to synthesize porphyrin rings (which make up the catalytic regions of enzymes in the electron transport chain of non-fermentative life forms) meaning that LAB are obligate fermentors (Salminen & von Wright 1998).

4.1.3 Lactic acid bacteria identification

Before the advent of molecular biological techniques for genetic identification of LAB, six major characteristics were used to identify the bacteria, namely microscopic analysis with the aid of a Gram stain, a catalase test, assessment of whether the isolate was homo- or heterofermentative, assessment of whether the isolate produces lactate under both aerobic and anaerobic conditions (obligate aerobic producers of lactic acid are not LAB), the types of carbohydrates which can be fermented and the isomers of lactate produced (Sneath *et al.* 1986). The fermentation profiles of LAB can be simply and effectively measured using the API 50 CH and API 50 CHL test strips manufactured by BioMerieux/API systems, France (Olsen *et al.* 1995, Gubag *et al.* 1996, Sanni *et al.* 1999). This system gives a tentative identification which has been used to assist in the identification of LAB in recent papers (Mathara *et al.* 2004, Thapa & Tamang 2004). The speed and ease of use in remote areas where DNA sequencing equipment is not available has necessitated the use of physiological and morphometric identification to characterise LAB in traditional beverages.

The small sub-unit 16S rRNA sequence, or specific sequences therein, are useful identifiers of bacteria (Woese 1987). In the subsequent decade and a half since the publication of the paper of Woese (1987), 16S rRNA analysis has led to the reassignment and renaming of many species and even genera of LAB, and the development of further genetic identification aids, such as the sequencing of the *pepT* and *pepV* (enzyme) genes, which has allowed even greater discrimination between closely related LAB (Mori *et al.* 2004). The analyses of phylogenetic trees drawn up with 16S rRNA sequence data and the sequences of other highly conserved regions has yielded similar trees leading to the conclusion that 16S rRNA phylogenies provides acceptable classification of LAB (Schleifer *et al.* 1995). Sequences may be compared against uploaded sequences at the Ribosomal Database Project (Larsen *et al.* 1993, Maidak *et al.* 1996).

In addition to molecular and basic physiological and morphometrical analysis, the observation of isolates of mixed cultures by scanning electron microscopy can yield additional information about LAB (Swaffield *et al.* 1997).

4.2 Objectives

To determine:

- The identity of LAB in *iQhilika*; and
- If the identified LAB contribute to spoilage, or product stability, or both.

4.3 Materials and methods

4.3.1 Samples

Two 250ml *iQhilika* samples were obtained from Mr M. Makwana in Grahamstown. Mr Makwana is a medium volume traditional brewer producing 60-80 litres per week, largely for sale in the informal tavern that he operates from his front veranda. The samples were taken from a batch of *iQhilika* which was near the end of its shelf life and was noticeably sour. Mr Makwana emphasised, as did all other traditional brewers, that the souring is not desirable and that the product should be fresh and clean tasting.

Mr Makwana had indicated that at the end of that fermentation, the biomass preparation (*imoela*) would be removed and poured through a canvas bag, after which it would be suspended under the eaves of his house and dried before reuse so as to reduce the level of sourness in the next batch. This is an important example of a traditional biotechnological solution to infection of the *imoela* which allows a means of reducing the severity of spoilage.

4.3.2 LAB isolation

LAB were separated from yeast and extraneous matter in the *iQhilika* using two centrifugation steps (4000 g and 10 000 g) similar to the method developed by Lonvaud-Funel & Joyeux (1994) for isolating *Leuconostoc* LAB from wine.

De Man-Rogossa-Sharpe (MRS) agar was developed for the isolation of LAB (de Man *et al.* 1960) and has been used by many researchers to isolate LAB from traditional fermented products (Sanni *et al.* 1999, Wachter *et al.* 2000, Arici & Coskun 2001, Mathara *et al.* 2004). This agar medium supplemented with cyclohexamide is

recommended by the European Brewery Convention for the detection of LAB (Sakamoto & Konings 2003).

In this investigation, the centrifuged LAB were re-suspended in filter sterilized *iQhilika*. The suspension was subjected to a 100 times serial dilution in filter sterilized *iQhilika* (0.22 µm Cameo cellulose acetate syringe filter). Aliquots (100 µl) were plated in duplicate onto MRS (Oxoid) agar plates and placed in anaerobic jars, together with one anaerobic gas pack (Oxoid) per jar. One set of plates were placed at 28°C and the other at 37°C and incubated for 48 hours.

Colonies isolated were examined microscopically and a number of colonies were found to be contaminated with yeast cells. The isolates were re-plated onto MRS (Oxoid) agar, which when sufficiently cool, had been supplemented to 0.01% wt/vol with sterile filtered cyclohexamide (0.22 µm Cameo cellulose acetate syringe filter). Filter sterilized cyclohexamide was added to MRS agar to inhibit yeasts in LAB studies (Kung *et al.* 2003).

A number of the isolates obtained from yeast contaminated colonies did not grow well once separated from the yeast. These colonies were cultivated by inoculating MRS cyclohexamide plates and then overlaying the plates with additional MRS cyclohexamide agar to facilitate a more anaerobic environment.

Isolates were re-examined for the presence of yeasts. Clean isolates were taken and streak plated onto MRS agar and the resulting representative colonies used to inoculate API 50CH (bioMerieux) strips using *Lactobacillus* media as per the manufacturers instructions. Isolates were scored after 24 and 48 hours and the results determined by the BioMerieux API 50CH database.

4.3.3 Electron microscopy

Samples were prepared as described in Chapter 2, section 2.3.1.4.

4.4 Results

4.4.1 Isolation

Eighteen colonies, representing a random sample of colonies growing on the agar plates, were isolated from the initial streak plants and named, A, B, C, D, E, F, G, H, I, J, K, L, M, BB1A, BB2A, BB1B, BB2B, BB6B. These colonies were sub-cultured onto fresh MRS agar plates.

4.4.2 Carbohydrate assimilation/utilization profiles

The API 50 CH (bioMerieux) system provided reasonably precise identifications for all but one of the isolates (Table 4.1)

Table 4.1 Tentative identifications of isolates based on carbon assimilation profiles. The percentages listed show the percentage match to a known strain for that isolate.

Isolate	Identification
A	<i>Lactobacillus curvatus</i> (99.9%)
B	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> (99.8%)
C	<i>Pediococcus damnosus</i> (81.5%)
D	<i>Lactobacillus curvatus</i> (99.8%)
E	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> (92.8)
F	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> (92.8%)
G	Unidentified
H	<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i> (78.4%)
I	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> (99.8%)
J	<i>Lactobacillus curvatus</i> (99.9%)
K	<i>Pediococcus damnosus</i> (96.8%)
L	<i>Lactobacillus curvatus</i> (81.1%)
M	<i>Lactobacillus curvatus</i> (99.8%)
BB1A	<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i> (83.5%)
BB2A	<i>Lactobacillus plantarum</i> (99.1%)
BB1B	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i> (99.7%)
BB2B	<i>Lactobacillus plantarum</i> (99.8%)
BB6B	<i>Lactobacillus acidophilus</i> (84.9%)

4.4.3 Electron microscopy

SEM observation of *imoela* showed that bacteria formed a significant proportion of the total biomass (Figures 4.1 & 4.2).

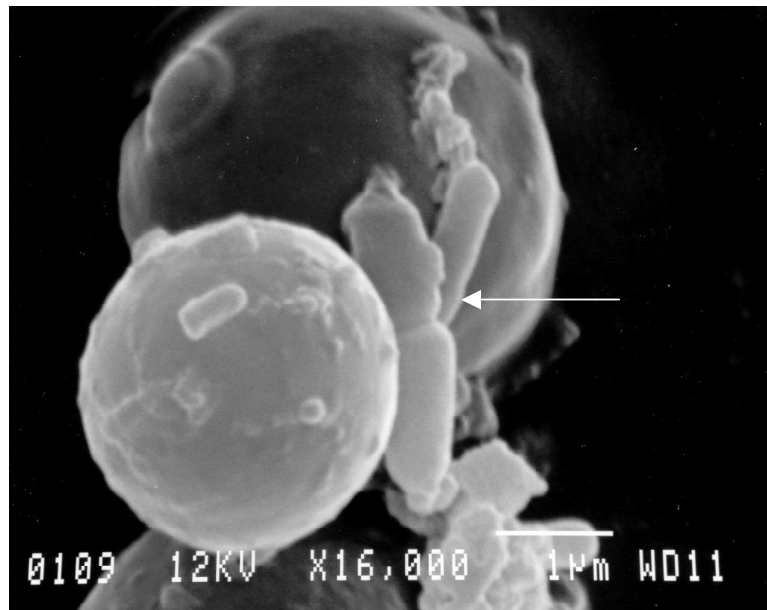


Figure 4. 1 A scanning electron micrograph (SEM) showing the presence of rod bacteria on the surface of the yeast within the traditional biomass preparation.

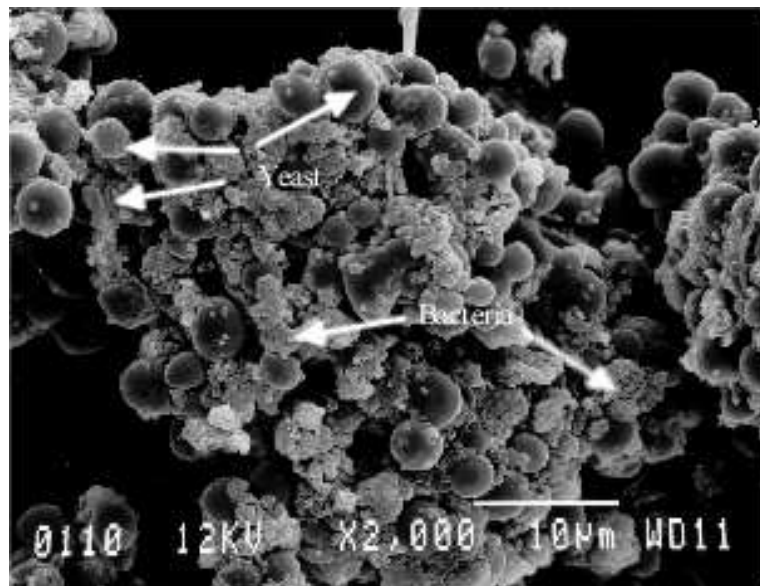


Figure 4. 2 This SEM shows a floc of yeast and bacteria. It is evident that although somewhat smaller in individual size, the bacterial biomass in the floc appears to be in the region of 25 to 30% of the total biomass.

4.5 Discussion

The presence of LAB in beverages can either be beneficial or detrimental to the product. *Pediococcus* and *Lactobacilli* are responsible for 70% of microbial beer spoilage incidents in commercial breweries (Sakamoto & Konings 2003). As is evident from Table (4.2) *Lactobacillus* sp. make up approximately 78% of the total isolates and *Pediococcus* sp. another 11%.

Table 4. 2 The frequency of the different LAB based on the identification of the random selection of isolates. (N=18)

Identification	% frequency
<i>Lactobacillus curvatus</i>	28
<i>Lactobacillus delbrueckii</i> subsp. <i>delbrueckii</i>	22
<i>Pediococcus damnosus</i>	11
Unidentified	5.5
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	5.5
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	11
<i>Lactobacillus plantarum</i>	11
<i>Lactobacillus acidophilus</i>	5.5

P. damnosus strains have been reported to contribute ropiness, as a result of their production of a sticky polymer of β -glucan (Li & Kong 2004, Walling *et al.* 2005). The production of this ropery characteristic is plasmid bound and not all strains of *P. damnosus* possess the ability to produce β -glucan (Walling *et al.* 2005). *P. damnosus* is a serious beer spoilage organism, and in addition to producing the ropery characteristics, also synthesizes large amounts of off-flavours and acids, which contribute an unpleasant taste and bouquet (Sakamoto & Konings 2003).

Following the outcome of this experiment, a sample of mead which had gone ropery was shown to two traditional brewers who both recognized the characteristic and had had experience of ropery *iQhilika*. It was also explained that this was considered to be unfortunate and undesirable.

Of the *Lactobacilli* isolated from *iQhilika*, *L. plantarum* and *L. curvatus* are both listed as serious beer spoilage organisms (Sakamoto & Konings 2003). The same authors list *L. paracasei* subsp. *paracasei* as a less serious beer spoilage organism. *L. delbrueckii* is mentioned specifically as not being a beer spoilage organism, and

strains of many of the common spoilage *Lactobacilli* species are reported to also not be spoilage organisms (Sakamoto & Konings 2003). *L. delbruekii* subsp. *delbruekii* is predominantly found in vegetable fermentations (Delley *et al.* 1990) – in this light it may be present from the early preparation of the root supports. *L. plantarum* is found in many traditional fermented products and is a beneficial enteric LAB (Johansson *et al.* 1995).

L. mesenteroides is a heterofermentative LAB producing both lactic and acetic acid and is frequently used in and isolated from vegetable fermentations (Bergqvist *et al.* 2005). Uzochukwu *et al.* (2001) showed that dextrans, contributing a cloudy hazy appearance to palm wine, were produced by LAB such as *L. mesenteroides*. *L. mesenteroides* has been regularly found in sugar refineries making brown sugar in South Africa (Eggleston *et al.* 2004). Brown sugar is normally added to *iQhilika* in urban areas when honey is scarce.

When questioning traditional brewers about sourness in beverages it was generally agreed that the less sour the *iQhilika* the better. Samples of *iQhilika* produced in the laboratory, without LAB, were given to traditional brewers and unanimously accepted as being desirable *iQhilika* samples.

Traditional brewers also mentioned that in damp weather the *imoela* yeast preparation failed to dry completely when hung out in a bag. This sometimes led to the *imoela* becoming mouldy. *Lactobacillus acidophilus* can exert a strong inhibitory effect on the growth of a number of common moulds (De Muynck *et al.* 2004). This may be beneficial to a limited extent, although the same authors noted that many researchers have found that the metabolites of LAB also inhibit yeasts. It is however important to note that the mode of *iQhilika* making has changed in recent years to include more and more sugar as a substitute for honey, given that honey is expensive and difficult to obtain. Certain honey samples inhibited the growth of *Lactobacillus acidophilus* through the production of hydrogen peroxide (Mundo *et al.* 2004). The same group noted inhibitory effects of honey against a range of different pathogens and food spoilage organisms. Serrano *et al.* (2004) reported low levels of glucose oxidase activity in two honey types frequently used in *iQhilika* production by traditional brewers, namely eucalyptus and citrus honey. It must be noted however that these honey sources are not indigenous to South Africa.

Scanning electron microscopy of the biofilms immobilised on *Trichodiadema intonsum* roots showed that a viscous mucilaginous compound entrapped cells within the biofilm. It is possible that this was produced by the yeasts, as observed by Vadasz *et al.* (2000), but it is likely that much of this mucilage was contributed by the bacteria growing within the biofilm.

4.6 Conclusion

iQhilika, like other traditional beverages, contains a large number of LAB. Some of these are known to cause spoilage in commercial beverages and two of the symptoms of this spoilage, ropiness caused by bacterial polymer production, and sourness symptomatic of organic acid production, were perceived as being undesirable outcomes by traditional brewers. It is possible that some of the LAB in *iQhilika* may contribute improved organoleptic properties when they are present in a specific batch, but their presence is not essential to the production of *iQhilika*. In the traditional environment, as with other LAB containing beverages, the consumption of the beverage enriches the gastro-intestinal tract with LAB, which may be beneficial as many LAB are probionts. In traditional *iQhilika* the presence of LAB may preclude the establishment of less desirable pathogenic spoilage organisms.

The objectives for this chapter have been met in that LAB were detected in *iQhilika* and identified. Some of the isolates were able to produce the symptoms of spoilage described by traditional brewers. It is likely that spoilage is also caused by many other groups of organisms, which can be studied in the future.

Chapter 5

Development of a bench-scale *iQhilika* production system

5.1 Introduction

Chapter 2 outlined research which showed that the traditional *iQhilika* bioreactor is a non-homogenous anaerobic batch tank bioreactor operated in a sequential batch configuration, with predominantly immobilised yeast that converts honey sugars to alcohol and carbon dioxide. It is operated in a sequential batch configuration with biomass retained between batches. A small amount of oxygen is absorbed by yeast cells at the air/*iQhilika* interface leading to increased efficiency of cells in this region as ethanol concentrations increase. Although the system was well mixed, stratification of the ethanol yield occurred with a lower yield closer to the air/mead interface as the fermentation initiated, and a higher yield at this interface as the fermentation neared completion.

In Chapter 3 it was evident that the dominant organoleptic properties and fermentation characteristics of *iQhilika* are due to the action of *Saccharomyces* yeasts selected through a traditional enrichment process.

In Chapter 4 it was however found that populations of spoilage organisms become established in the yeast preparation that contributed a sour flavour to the *iQhilika* and certain undesirable characteristics.

In contrast to the 48 hour 7 % alcohol yield fermentation in the traditional *iQhilika* bioreactor, conventional commercial and laboratory-scale mead making is characterised by slow fermentations taking weeks or months to ferment (Steinkraus & Morse 1966) yielding products with 6.4% to 16.6% alcohol (Steinkraus & Morse 1973).

The problem of contamination in mead making is overcome by heating and flash heating (Kime *et al.* 1991 a), sulfiting (Karuwanna *et al.* 1993) or in more recent years, ultrafiltration (Kime *et al.* 1991 a & b). In addition to removing contaminants from mead, the heating process can remove proteins which can cause later product

stability problems but also damages the organoleptic properties of the mead (Kime *et al.* 1991 a & b). An alternative to heating to remove contaminants and proteins is ultrafiltration (Kime *et al.* 1991 a & b).

To improve the efficiency of mead fermentation, salts and co-factors have been added (Steinkraus & Morse 1966) and/or fruit juices to provide additional nutrients (Steinkraus & Morse 1966, Karuwanna *et al.* 1993). Various methods have been used to improve mass transfer in mead bioreactors such as agitation and have been patented in the past (Steinkraus & Morse 1966).

The traditional *iQhilika* bioreactor has overcome the problem of slow fermentation through the inclusion of brood and/or pollen as a nutrient source. The problems of inefficient mass transfer are overcome by a simple immobilisation support that floats at various levels. The single largest shortcoming of the *iQhilika* bioreactor is however contamination by undesirable spoilage organisms.

In the light of these observations it is necessary to review the following topics to explain the objectives of this chapter.

5.1.1 Immobilised biomass, glycerol production and flavour

In terms of beverage production, immobilised biomass has certain advantages and certain disadvantages. After alcohol, glycerol is the major product of fermentation (Balli *et al.* 2003, Lubbers *et al.* 2001, Omori *et al.* 1997). In fermentations involving *Saccharomyces* it is produced by the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is then reduced to glycerol (Lubbers *et al.* 2001, Sahoo & Agarwal 2001, Wang *et al.* 2001). It is an important chemical influencing flavour in many different beverages such as wine (Lubbers *et al.* 2001, Balli *et al.* 2003), *sake* and *socchu* (Omori *et al.* 1997). Noble & Bursick (1984) found that glycerol influenced the perceived viscosity and sweetness in sweet white wines. Lubbers *et al.* (2001) found through a set of experiments using model wines that glycerol had no effect on the typical wine volatile flavour compounds examined.

Immobilisation of wine yeasts can cause production of greater glycerol concentrations (Balli *et al.* 2003). In a series of experiments free yeasts produced

10.2-12.8 g/l glycerol whereas immobilised yeasts produced 11.9-14.9 g/l glycerol (Balli *et al.* 2003).

An advantage of the traditional *iQhilika* system is that it employs immobilised biomass, which may have an effect on glycerol production and enhance the overall flavour and sensory characteristics of the liquor. In designing an *iQhilika* production system it would be important to retain the immobilised biomass aspect of production, as this would ensure product similarity. Steinkraus & Morse (1966) reported on their, as well as many other researchers', efforts to increase the flavour and body of mead. The lack of these characteristics in mead at the time were identified as marketing hurdles. Honey generally has a subtle bouquet. Mead with a higher concentration of glycerol would theoretically have a greater potential for human perception of these subtle flavours.

5.1.2 Honey storage

Mundo *et al.* (2004) mentioned that honey has a generally low microbial density and has a long shelf life. Zamora & Chirife (2004) explained that honey generally consists of a mixture of glucose and fructose and smaller quantities of trace sugars. Solutions with high water activities are very effective preservatives and can be sufficient to inhibit even osmotolerant yeasts (Maltini *et al.* 2003). If however the supersaturated sugars should crystallize, they would sediment and the change in water activity would allow yeast growth in a watery layer on top of the crystal layer (Snowden & Cliver 1996). This can be avoided by stirring the honey as it crystallizes. Beekeepers frequently keep crystallised honey in sealed drums for up to five years without loss in quality.

Fruits such as grapes and apples have shelf lives measured in weeks. In contrast to mead making where honey is merely dissolved, the making of wine or cider requires the fruits to be juiced and the juices or musts fermented immediately. As a consequence of this it is logical that the vast majority of wines and ciders would be produced in batch systems. The choice of a batch system for mead making is, in the light of the storage potential of honey, not the only system, with continuous fermentation a potential alternative. Another example of a storable fermentable carbohydrate source is malted grain.

5.1.3 Continuous fermentation

Continuous fermentation is useful where substrate can be stored, such as barley for beer (Royston 1966). Honey can also be stored, hence continuous fermentation of mead is a possibility.

5.1.3.1 Continuous fermentation of beer

The following is a summary of the guidelines listed by Royston (1966) for a continuous beer bioreactor:

- 1) It should precisely reproduce the same conditions that occur in a batch process so that the same minor by-products are produced as these are important for flavour.
- 2) The process should be more rapid than the batch process so that even with the more sophisticated plant requirements a net improvement in economy of operation is achieved.
- 3) The process should be able to run for at least 6 months free from mechanical problems or infection.

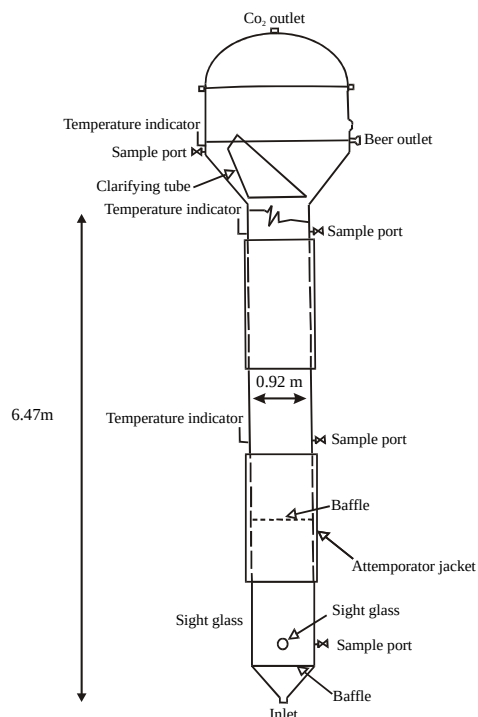


Figure 5. 1 The tower bioreactor used by Royston (1966).

The continuous tower system of Royston (1966) shown in Figure 5.1 operated using flocculent yeast strains which settled into the base of the tower, close to the inlet. Wort was pumped through at a constant rate and a heterogeneous fermentation profile was achieved for the bioreactor, with a rapid decline in specific gravity in the area at the base of the tower with the highest yeast concentration. Once clear of the yeast bed, residual yeast settled back into the bed and low cell density product flowed to the top of the bioreactor. The expanded head region of the bioreactor served to act as an additional settler with gas rising in the middle and yeast settling out in the still expanded region and then flowing down the side of the expander due to gravity and returning to the yeast bed.

Royston (1966) mentioned that in this system, the ethanol yield is some 3% higher than from a batch bioreactor. This is attributed in part to the lower cell growth rates in the bioreactor.

If a tower bioreactor were to ferment an extremely expensive substrate with the objective of making an alcoholic beverage, this would translate into a considerable economic advantage. Instead of converting expensive honey into low value yeast waste and product, the expensive honey could be almost entirely converted into product.

An extensive search of the literature yielded no published examples of continuous bioreactors designed for the production of mead from honey. However a number of creative configurations of continuous bioreactors used for fermenting various sugar solutions were found. Examples of the use of these bioreactors to produce alcohol from sugar rich molasses, corn syrup, and fruit juices are reviewed in the ensuing pages.

5.1.3.2 Molasses and corn steep liquor

Chen *et al.* (1994) used a packed bed tower bioreactor with self aggregating *Saccharomyces uvarum* to convert molasses or corn syrup sugars to ethanol. The bioreactor design employed (Figure 5.2) was similar to that of Royston (1966), with a tubular section and a settler region, with the main difference being that where

Royston (1966) used a riser to separate bubbles from medium, Chen *et al.* (1994) used a mesh in the settler.

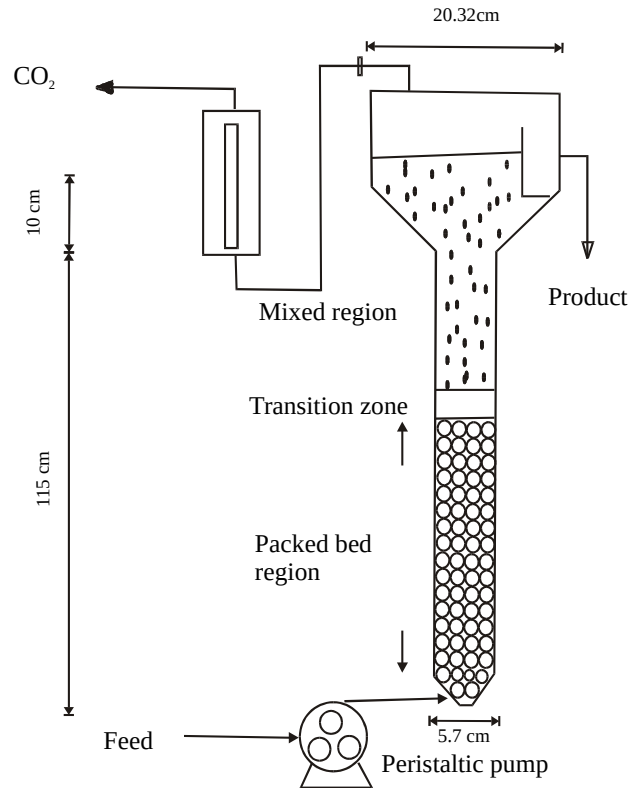


Figure 5.2 The continuous tower bioreactor of Chen *et al.* (1994). Note the three zones in the tower. The first is the packed bed with yeast immobilised in large granular flocs. The second is the transition zone. The third is the mixed region where yeast flocs are fluidised by gas escaping from the packed bed.

The highest ethanol concentration in effluent achieved was 12.1% from an initial sugar feed concentration of 180 g/l. This gives a yield of 0.67 g ethanol per g of sugar which is higher than the 0.51 g yield which is mentioned by the authors to be the theoretical maximum. One of the explanations for this phenomenon was that sugars contributed by the corn steep liquor added to the media increased the overall sugar availability.

The bioreactor used by Chen *et al.* (1994) was stable for 3 months with no loss of fermentative capacity when it was shut down at the end of this period.

5.1.3.3 Cider

Nedovic *et al.* (2000) devised a system of achieving both of the important fermentation processes for cider in a co-immobilised dual biocatalyst continuous system. The first converted the fruit sugars to alcohol with *Saccharomyces bayanus* and the second concerned the maturation fermentation of malic acid to lactic acid by *Leuconostoc oenos* (Figure 5.3).

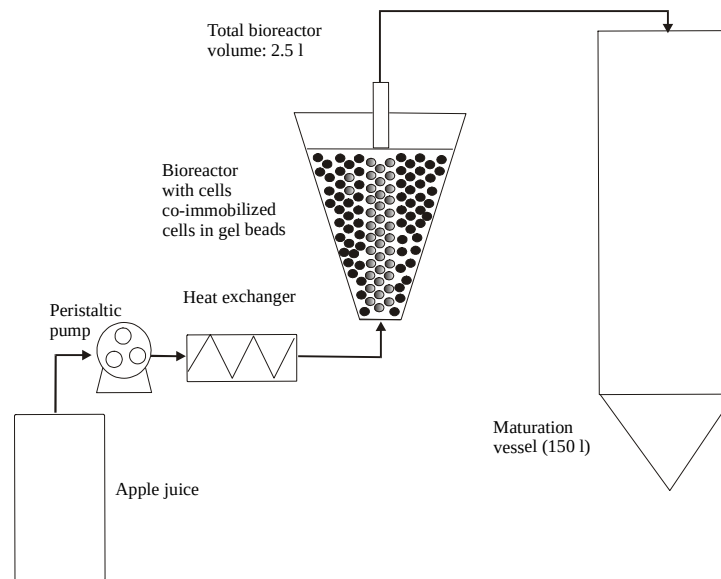


Figure 5.3 The experimental set-up used by Nedovic *et al.* (2000), to perform a continuous fermentation with apple juice and immobilised cells of *S.bayanus* and *L.oenos*.

Unlike Chen *et al.* (1994), Nedovic *et al.* (2000), set out to develop a system for the production of a beverage to be consumed by humans. As with the bioreactor of Royston (1966), the bioreactor of Nedovic *et al.* (2000), produced an acceptable product with characteristics largely similar to the batch produced product, but slight differences were evident due to the changed physiological state of cells in continuous bioreactors. They referred to this physiological state as a pseudo-stationary phase given that little cell growth occurred. This decrease in cell growth is notable if one considers that de la Roza *et al.* (2003) report a 10 fold increase in cell concentration in commercial and laboratory batch cider fermentations within the first 5 hours of fermentation.

5.1.3.4 Wine production

Kourkoutas *et al.* (2002 a), produced wines using *Saccharomyces cerevisiae* immobilised on pieces of apple in a continuous bioreactor. The apple cuts were

placed in a tubular bioreactor and grape must passed through the tube in an upward direction, with the bioreactor being operated at a number of different temperatures. The system produced acceptable wines which had flavour and aromatic properties comparable to, or, in some cases, better than equivalent batch wines. Ethanol productivities were also markedly higher than batch systems. The same authors performed similar experiments (Kourkoutas *et al.* 2002 b) using quince cuts and achieved similarly satisfactory results. The main reason for choosing fruit pieces was that they are inexpensive and are considered to be food grade. This has many advantages over other systems using synthetic or semi synthetic immobilization matrices.

5.1.3.5 Other continuous fermentation systems

Many other examples of continuous bioreactors for processing fruit juices and plant saps have been published. Nigam (2000) used κ -carrageenan immobilised *Saccharomyces cerevisiae* ATCC 24553 cells to ferment pineapple cannery waste. This system was 11.5 times more efficient than a free cell system. Wehndhausen *et al.* (2001) used *Saccharomyces sp.* immobilised on an inorganic support to produce ethanol from sugar cane syrup, while Nigam *et al.* (1998) fermented cane molasses with yeast cells immobilised in an agar matrix. Lactose sugar and its hydrolysates, common waste products of the dairy industry have been fermented using flocculating *S.cerevisiae* capable of converting these sugars to ethanol (Terrel *et al.* 1984, Dominguez *et al.* 1999). Many complex carbon sources have been hydrolysed and used as sugar sources for continuous fermentation, specifically for ethanol production. Krishnan *et al.* (2000) showed that corn starch could be hydrolysed and fermented in a set-up consisting of a continuous immobilised enzyme system, followed by an immobilized *Zymomonas mobilis* ethanol fermentation. Palmqvist *et al.* (1998) employed both batch and continuous fermentation systems to ferment spruce wood hydrolysates for fuel ethanol production.

A feature of the systems described for continuous fermentation is that they were generally operated for long periods of time and were resistant to infection by unwanted microbes (Royston 1966, Admassu *et al.* 1985, Chen *et al.* 1994).

The result is that a large body of literature exists on continuous fermentations which provide excellent guidelines for designing a continuous system to ferment mead.

5.1.3.6 Potential for the continuous fermentation of *iQhilika*

The traditional *iQhilika* bioreactor is an immobilised biomass batch system (Chapter 2). The traditional bioreactor consists of a packed bed of roots with biomass attached. The system appears to closely resembles packed bed regions of the continuous configurations of Royston (1966), Chen *et al.* (1994), Nedovic *et al.* (2000) and Kourkoutas *et al.* (2002 a & b) and it is quite possible that operating the traditional bioreactor in a batch configuration reduces its efficiency due to mixing problems.

Continuous operation, if coupled with increased efficiency and reduced incidence of infection by spoilage organisms, would improve process economics. In order to operate the *iQhilika* immobilised biomass bioreactor in a continuous mode, the bioreactor would need to be redesigned to allow this form of operation.

This chapter reports on the design and operation of a bench-scale continuous *iQhilika* production system which produces a product with high fidelity to the traditional product, but which optimally employs the inherent advantages of the traditional immobilisation support.

5.2 Objectives

The objectives included:

- Designing and operating a continuous *iQhilika* bioreactor
- Characterising the bioreactor

5.3 Materials and methods

5.3.1 Bioreactor design

A glass tower bioreactor was constructed to the dimensions in Figure 5.4 using Pyrex glass.

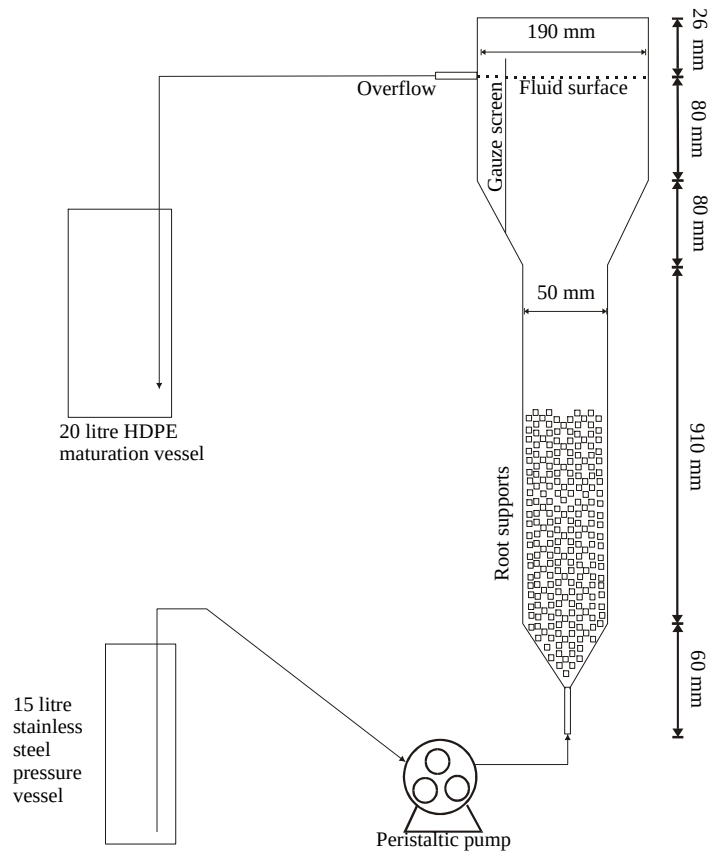


Figure 5. 4 The glass tower bioreactor. The 20 litre high density polyethylene drum (HDPE) and the 15 litre stainless steel pressure vessels were replaced with fresh vessels as they filled and emptied respectively.

5.3.2 Support configuration

The function of the *Trichodiadema intonsum* support, as demonstrated in Chapter 4, was considered to be to immobilise a certain amount of yeast in a thin biofilm. In order for the support to have optimal functionality it is important to know what the smallest support particle size is which will have a settling velocity sufficient to retain it in the packed bed region of the bioreactor.

Sections of imoela root were cut into cubes 2 mm, 5 mm and 10 mm in size. Ten cubes of uniform weight of each size were selected. The tower bioreactor vessel detailed in Figure 5.4 was filled with sugar solutions of specific gravities 1.030 and 1.050. An air stone was placed in the base of the bioreactor vessel such that carbon dioxide gas could be bubbled through the column at flow rates of 0, 1 and 2 l/min.

Cubes of support material were dropped into the top of the bioreactor and the time taken to settle to the air stone was measured using a stop watch.

5.3.3 Inoculum and support preparation

A pure culture of strain F (Chapter 3), which was selected by traditional brewers as being the optimal *iQhilika* strain, was used to inoculate flasks containing 2 litres of growth media [1.2 g yeast nutrient (Ecolab), 1.4 g yeast extract (Difco), 20 g glucose per litre (Merck)] and placed on an orbital shaker at 100 rpm for 48 hours at 28°C. As the signs of fermentation in the flasks lessened (± 120 h) as nutrients were depleted, the cultures were allowed to settle at 4°C for 8 hours. The concentrated cell slurry was centrifuged at 2500 g for 15 minutes and the separated wet pellets weighed.

Sufficient 2mm cubes of *Trichodiadema intonsum* roots with an average mass of 0.03 g each were cut to displace a volume of 0.5 l. The support preparation was placed in a 5 l flask and the 490 g of wet previously prepared yeast cake was added to this mixture together with 3 l of fresh media. The culture was placed in a controlled environment room at 28°C for 3 days. The culture was aerated with filtered air at a flow rate of 1 l/min. After 3 days the culture was placed at 4°C for 24 hours and the clear spent media poured off.

5.3.4 Yeast immobilisation and growth

The reviews of various continuous systems in the introduction of this chapter show that the retention of the yeast biomass in the packed bed region is important in terms of retaining biocatalyst and maintaining efficiency. In order to operate an *iQhilika* bioreactor it would be important to understand the triggers which result in cells flocculating and attaching to the imoela immobilization matrix.

A colony of strain F was inoculated into 100 ml of growth media [1.2 g yeast nutrient (Ecolab), 1.4 g yeast extract (Difco), 20 g glucose per litre (Merck)] and placed on an orbital shaker at 100 rpm for 24 hours at 28°C. At this point the cells had not flocculated. The culture was found to have an OD₆₀₀ of 0.11.

Growth medium was prepared for the following concentrations of the sugars. Glucose (Merck) 12.5 g/l, 25 g/l, 50 g/l and 100 g/l. Fructose (Merck) 12.5 g/l, 25 g/l, 50 g/l and 100 g/l. A theoretical honey solution comprising 50% fructose and 50% glucose as solids was used to prepare media with 12.5 g/l, 25 g/l, 50 g/l and 100 g/l of honey sugars. Honey typically consists of a mixture of glucose and fructose with traces of sucrose (Fallico *et al.* 2004, Fernandez-Torres *et al.* 2004), minerals (Rashed & Soltan 2004, Terrab *et al.* 2004), proteins/amino acids (Hermosin *et al.* 2003), lipids (Barhate *et al.* 2003) and other smaller flavour compounds (Toth *et al.* 1987, Galan-Soldevilla *et al.* 2005). Due to the reported variability in these characteristics the option of using a simulated honey sugar solution was used with equal parts of the two major honey sugars, glucose and fructose. Growth media was adjusted to pH 3.8 with 2M Tartaric acid (Robertsons) and 2M KOH (Alpha).

The growth media for different sugar concentrations were filter sterilized (Cameo 0.22 µm cellulose acetate syringe filter) and 300 µl was auto-pipetted into each well of a 96 well micro titre plate (Merck). All wells were inoculated with log phase (OD₆₀₀ of 0.15) strain F yeast. 10 µl of light mineral oil was placed over all wells to limit evaporation and allow semi anaerobic conditions to prevail in the well.

The micro titre plate was placed in a Bio-Tek Instruments Powerwave X. The Powerwave X instrument was controlled using K-C Junior V 1.31.2 Software. The reader was set to incubate the plate at 25°C. Readings were taken at 600 nm every half an hour for 43.5 hours.

The point at which visible floc formation began was noted.

5.3.5 Electron microscopy of flocs

Floc samples were processed as in Chapter 2, section 2.3.1.4.

5.3.6 Bioreactor stocking

The tower bioreactor was sterilised using a 4% formaldehyde solution for 24 hours, then rinsed with two volumes of sterile autoclaved deionised water. When draining the column, sterile filtered air was allowed to replace the liquid volume removed.

The exterior of the column and surrounding areas were sprayed with 70% ethanol until wet and allowed to dry. The bung in the top of the bioreactor was removed and the inoculated support material prepared in section 5.3.2 was poured into the bioreactor vessel.

5.3.7 Bioreactor biomass development cycle

To encourage biomass to form on the supports an additional 2 litres of growth medium containing 30 g/l glucose, 0.67 g/l yeast nutrient (Ecolab) and 0.7 g/l yeast extract was recycled through the bioreactor for 72 hours. During this time visual observations were made on the activity of biomass in the bioreactor.

After 72 hours 2 litres of fresh growth medium was pumped through the tower at a rate of 300 ml/h.

5.3.8 Bioreactor operation

Must for fermentation was prepared by dissolving Eastern Cape wild flower honey (produced by Makana Meadery at Mosslands farm near Grahamstown) in untreated water from Jamieson municipal reservoir, Grahamstown, until the desired specific gravity was obtained (Figure 5.5). The water had the characteristics listed in Table 5.1.

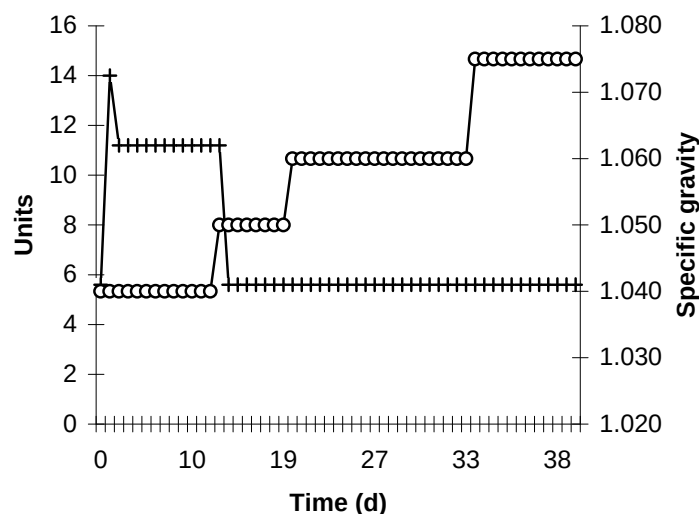


Figure 5.5 The glass tower bioreactor was operated at the flow rates in litres per day (+) with inflow specific gravities (o) as shown in this graph. The objective of this experimental run was to find the optimal flow rate and specific gravity at which the bioreactor could operate stably.

Table 5.1 An analysis of Jamieson Reservoir water, Grahamstown (Municipal Data).

pH	6.5 – 7
Colour (Pt - Co units)	129
Turbidity	0.6 – 1
Total Dissolved Solids (mg/l)	90-200
Total alkalinity as CaCO ₃ (mg/l)	9-11
Bicarbonate (mg/l)	11
Non-carbonate hardness (mg/l)	59-62
Sodium (mg/l)	20-37
Potassium (mg/l)	1-3.3
Chloride (mg/l)	40-76
Sulphate (mg/l)	30-69
Fluoride (mg/l)	0.5

Steinkraus and Morse (1966) added 1.0 g/l ammonium sulphate, 0.5 g/l potassium phosphate, 0.2 g/l magnesium chloride, 0.05 g/l sodium hydrogen sulphate and 5.0 g/l citric acid to a honey base and supplement mead must with this in various ratios. As is evident from the water analysis (Table 5.1) chloride is already present in the water, hence magnesium sulphate was substituted for magnesium chloride. Sodium reduces the flocculence of yeast, hence potassium salts were substituted for sodium

salts. Calcium assists in yeast flocculation hence the addition of calcium chloride. The following salts were added per litre:

7 mg/l	CaCl ₂ .2H ₂ O (Merck)
32 mg/l	MgSO ₄ .7H ₂ O (Merck)
32 mg/l	KH ₂ PO ₄ (Merck)
70 mg/l	(NH ₄) ₂ .SO ₄ (Merck)

In mead making recipes both citric and tartaric acid are used to control pH of the must (Acton & Duncan 1984). Food grade tartaric acid (Robertsons) was added at the ratio of 0.1 g/l. If necessary pH was adjusted to 3.7 with food grade KOH (Alpha).

Pollen, gathered by bees from wild flowers in the Grahamstown area in September 2001 was added to the must at rate of 0.5 g/l. The 10 g of pollen needed for a batch was first placed in a beaker and 50 ml of must added to it, whereupon it was stirred with a glass rod to break up the pollen pellets and poured through a sieve to remove coarse objects such as the bee parts and ants often found in pollen traps. The pollen mixture was then added to the majority of the must and mixed in. The must was sterilized by placing in a pressure vessel and autoclaving for 30 minutes at 120 °C then allowed to cool.

The must was pumped into the tower bioreactor using a variable speed Watson Marlow peristaltic pump. The *iQhilika* produced was placed in 20 litre maturation vessels for two weeks, after which the residual fermentation had ceased and the small amount of yeast in the mead had sedimented. The moderately clear mead was racked into fresh 20 litre drums and allowed to mature for 1-6 months before tasting. Some samples were kept for 2 years.

Samples were taken at regular intervals and observations of the dimensions of the yeast bed were made.

Michele & Hempel (2002) explain the difficulties in calculating liquid and gas hold up in bubble columns. In the light of the complexity of these systems, the following strategy was devised to obtain basic information from the glass tower bioreactor.

After 1000 hours of operation a 0.2 mm internal diameter high density polyethylene tube was lowered into the bioreactor and 2 ml samples of must removed from 0 cm, 45cm, 60cm, 90 cm and 120 cm. The dimensions of the yeast bed were measured and recorded.

After these samples had been taken the peristaltic feed pump was turned off and the bioreactor was left for a week to become completely inactive. The dimensions of the yeast bed in its dormant, gas free state were measured and the total volume of the bioreactor which was filled by liquid and yeast was measured in order to determine the gas hold-up of the bioreactor.

5.3.9 Sample analysis

HPLC Analysis

Samples were processed by HPLC as in Chapter 2, section 2.3.1.3.

Ethanol yields were calculated as grams ethanol produced per gram sugar consumed (Dharmadhikari 1999) using the following formula:

$$\text{Ethanol yield} = \frac{\text{g EtOH}}{\text{g Sugar}} \quad \text{Formula 5. 1}$$

Ethanol productivity was calculated using the following formula (Chen *et al.* 1994):

$$\text{Ethanol productivity} = \frac{\text{g EtOH} \times \text{Flow rate}}{\text{Liquid bioreactor vol.} + 24} \quad \text{Formula 5. 2}$$

The volumetric productivity for the entire reactor was calculated in the same way, except that the liquid volume was replaced by the total volume including headspace.

5.4 Results

5.4.1 Support density

Root samples were weighed and found to have an average density of 1.125 g/cm^3 . Hence a root particle would be able to sink in a solution of up to 244 g/l of honey sugars based on specific gravity.

5.4.2 Support configuration

The following graphs show the results of the experiments to determine the optimal support size.

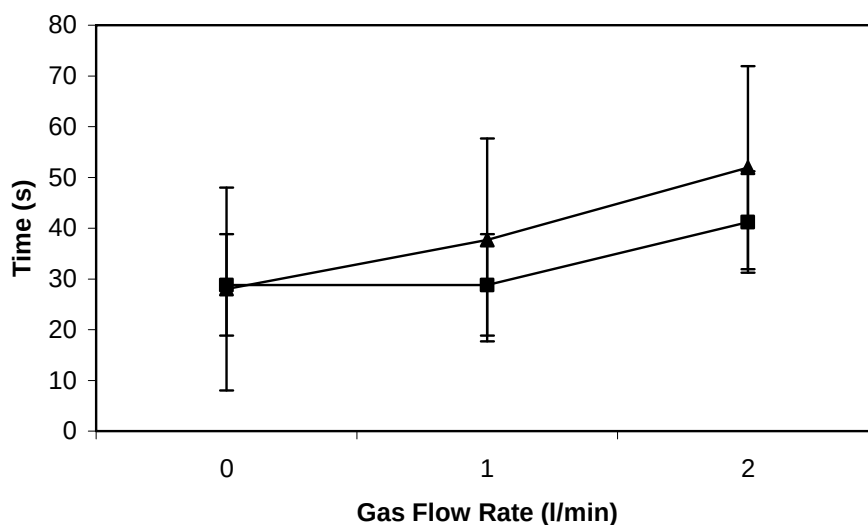


Figure 5. 6 Settling velocities obtained for 2mm cubic support particles at various carbon dioxide gas flow rates and specific gravities of 1.030 (▲) and 1.050 (■). Each point represents an average for 10 repeats and the standard deviation bars show the reasonably high variation in settling velocities obtained for this support size.

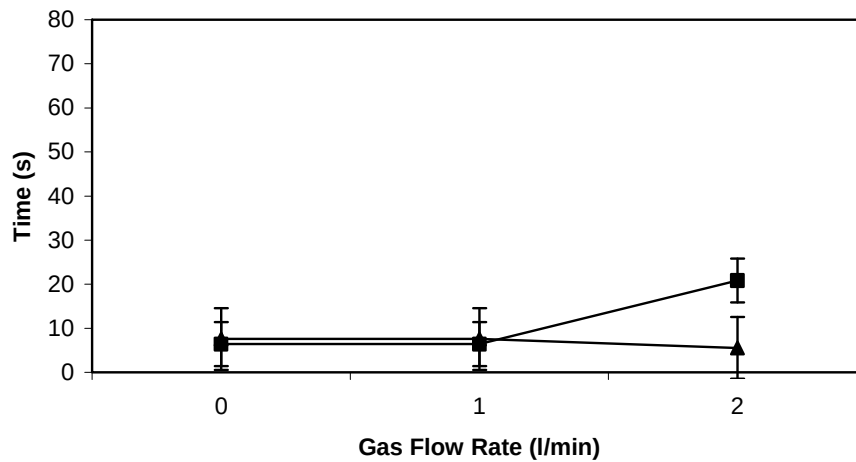


Figure 5. 7 Settling velocities obtained for 5mm cubic support particles at various carbon dioxide gas flow rates and specific gravities of 1.030 (▲) and 1.050 (■). Each point represents an average for 10 repeats and the standard deviation bars show the moderate variation in settling velocities obtained for this support size.

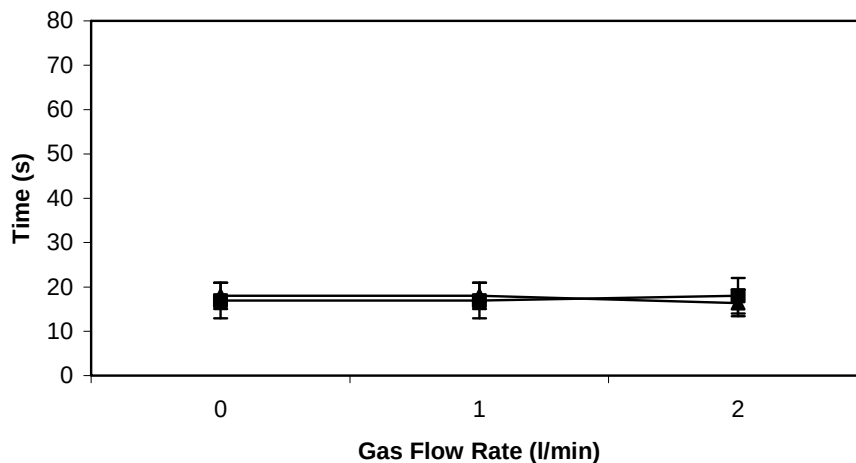


Figure 5. 8 Settling velocities obtained for 10mm cubic support particles at various carbon dioxide gas flow rates and specific gravities of 1.030 (▲) and 1.050 (■). Each point represents an average for 10 repeats and the standard deviation bars show the low variation in settling velocities obtained for this support size.

From the data in Figures 5.6-5.8 it was evident that although a 2 mm cubed particle settled more slowly than the other sizes, it was sufficiently capable of returning to the yeast bed even under conditions of high gas evolution.

Figure 5.9 shows the different stages of root support preparation.



Figure 5. 9 The *Trichodiadema intonsum* plant, left, showing the large taproot, as well as the peeled roots in the middle and cubed roots in the beaker on the right. Note the fermentation in the beaker occurred spontaneously minutes after the roots had been cubed and only water had been added.

5.4.3 Flocculation characteristics of strain F

The following graphs show the flocculation of strain F when grown on glucose, fructose or both.

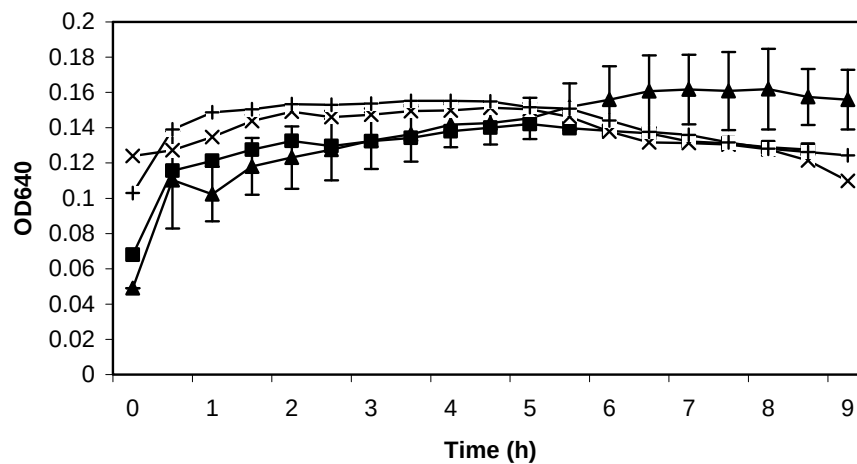


Figure 5. 10 Growth of strain F on glucose. For simplicity standard deviation bars have only been set for the 100 g/l experiment. 100 g/l (▲), 50 g/l (■), 25 g/l (X) and 12.5 g/l (+).

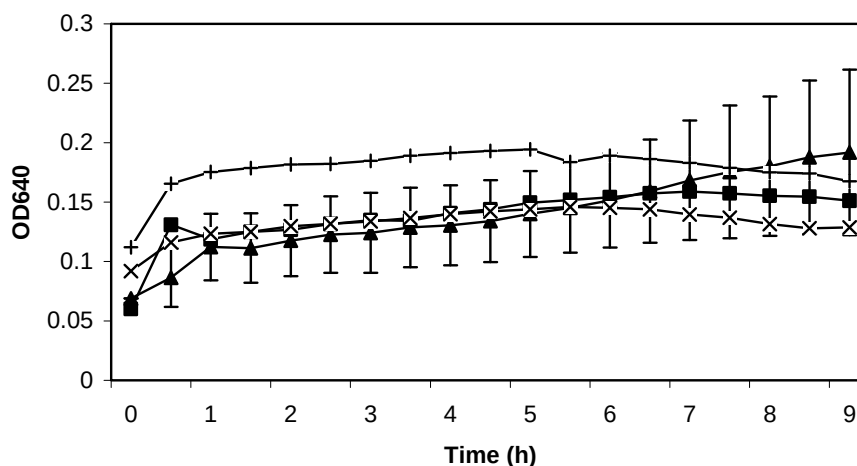


Figure 5. 11 Growth of strain F on fructose. Note standard deviation only shown for 100 g/l. Fructose concentrations of 100 g/l (▲), 50 g/l (■), 25 g/l (X) and 12.5 g/l (+). All concentrations showed moderate flocculation after 2 hours.

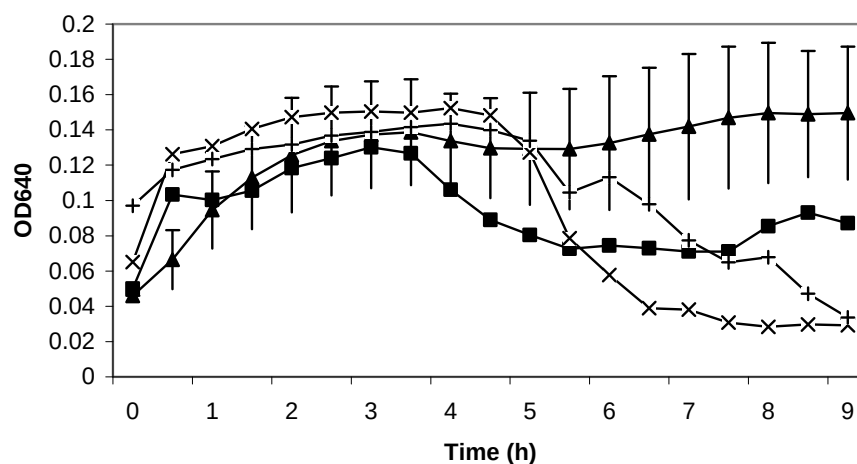


Figure 5. 12 Honey growth curves for strain F. Note standard deviation only shown for 100 g/l for simplicity. Honey sugar concentrations of 100 g/l (▲), 50 g/l (■), 25 g/l (X) and 12.5 g/l (+). Flocculation began occurring for all sugar concentrations at 3.5 hours, but was the most pronounced for low sugar concentrations.

The flocculation of cells is seen in the decrease in the absorbency of the liquid in the wells in the micro titre plates cause by cells clumping in flocs and permitting more light through in the gaps between flocs.

5.4.4 Electron microscopy of flocs

Figure 5.13 shows a section of flocculated strain F yeast.

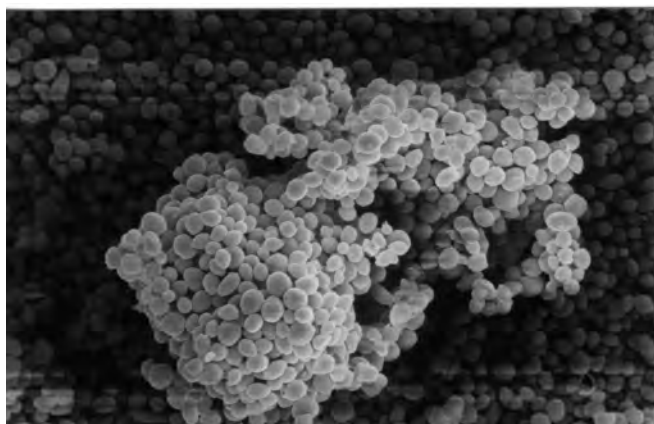


Figure 5. 13 A scanning electron micrograph (SEM) of a clump of flocculated strain F cells. Unlike the wild type flocs seen in Chapter 4, Figure 4.2, the cells in this floc are free of bacteria and largely free of extracellular polymeric substances, with adhesion being more likely due to clumping of cells by direct contact.

5.4.5 Bioreactor operation

The following pictures show the bioreactor in operation.

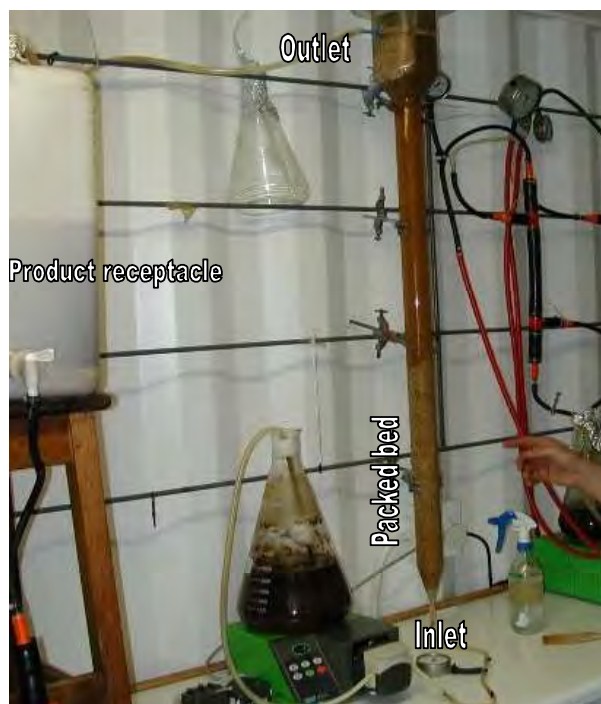


Figure 5. 14 The glass tower bioreactor in operation. (Photograph J. Cambray)



Figure 5. 15 The packed bed region of the glass tower bioreactor. Note how the yeast has formed semi-spherical biofilm particles around the 2 mm cubed root particles. The gas which evolved as a function of fermentation is visible in the spaces between supports. (Photograph J. Cambray)



Figure 5. 16 The transitional zone at the top of the packed bed, where support particles are fluidised by rising carbon dioxide bubbles. (Photograph J. Cambray)

The following graphs show the data derived from the operation of the glass tower bioreactor.

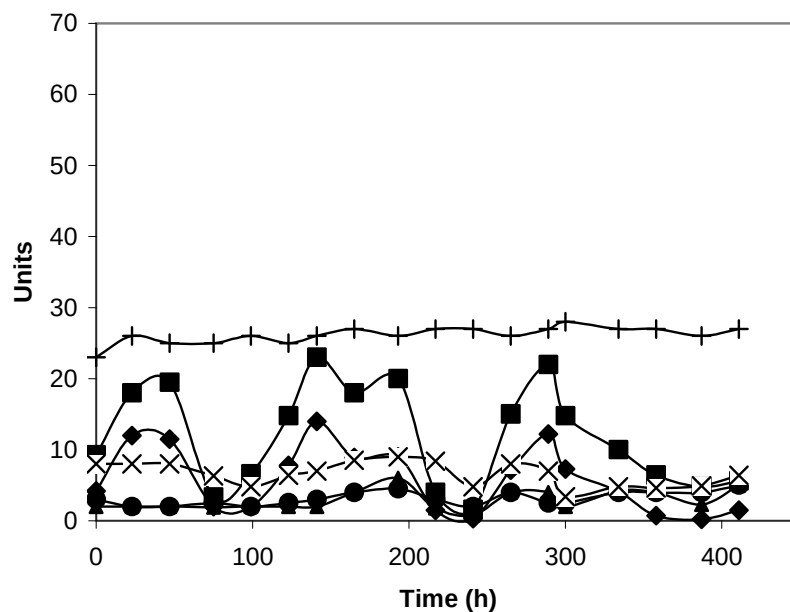


Figure 5.17 Operation parameters as well as effluent *iQhilika* characteristics for the first 411 hours. (●) Ethanol (% vol.), (◆) Glucose(g/l), (■) Fructose(g/l), (▲) Sucrose(g/l), (X) Glycerol (% vol.), Flow rate l/d (□), Specific gravity (-). The values for sugars are for the residual sugar in the effluent. Samples of influent were taken and used for calculations later in this chapter, however influent sugar concentrations are not shown here.

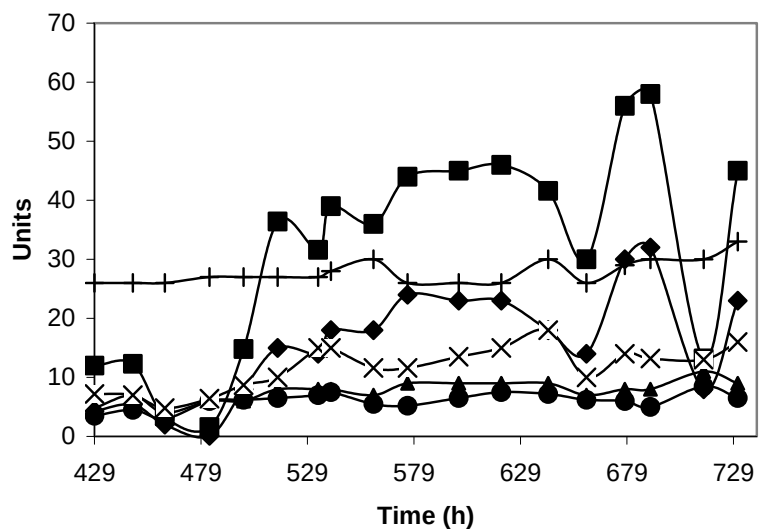


Figure 5.18 Operation parameters as well as effluent *iQhilika* characteristics for next 302 hours. (●) Ethanol (% vol.), (◆) Glucose(g/l), (■) Fructose(g/l), (▲) Sucrose(g/l), (X) Glycerol (% vol.), Flow rate l/d (□), Specific gravity (-). The values for sugars are for the residual sugar in the effluent. Samples of influent were taken and used for calculations later in this chapter, however influent sugar concentrations are not shown here.

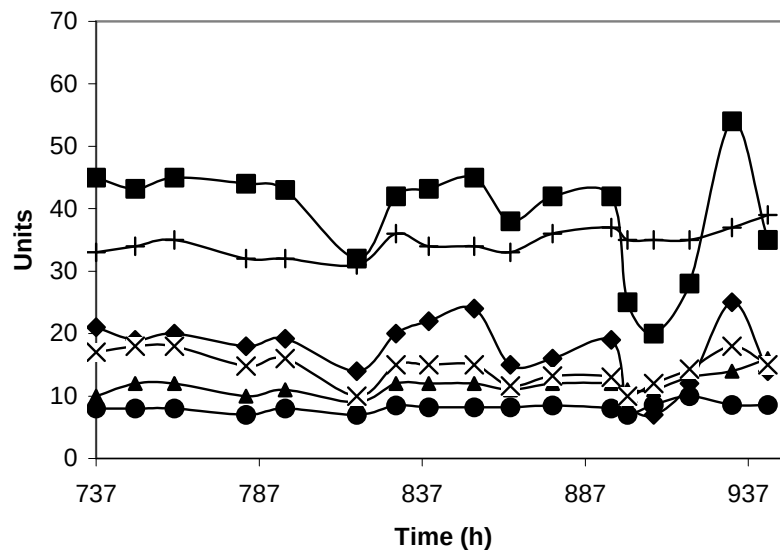


Figure 5. 19 Operation parameters (Specific Gravity of must and flow rate) and measured *iQhilika* characteristics for the final 206 hours of operation. (●) Ethanol (% vol.), (◆) Glucose(g/l), (■) Fructose(g/l), (▲) Sucrose(g/l), (X) Glycerol (% vol.), Bed depth (+), Flow rate l/d (□), Specific gravity (-). The values for sugars are for the residual sugar in the effluent. Samples of influent were taken and used for calculations later in this chapter, however influent sugar concentrations are not shown here.

5.4.6 Bioreactor fermentation profile

Visual observations of the bioreactor showed a gradual increase in gas volume across the depth of the yeast bed. The following profiles (Figures 5.20 and 5.21) show the corresponding increase in ethanol and decrease in sugar concentration across the profile of the glass tower bioreactor.

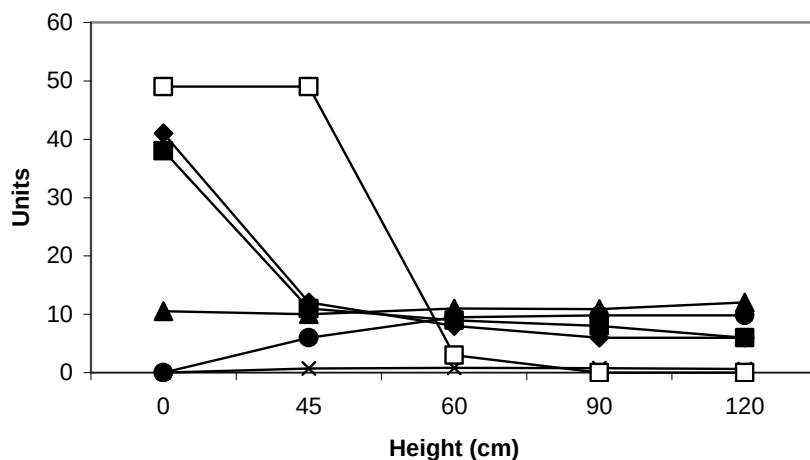


Figure 5.20 The depth profile of the tower glass bioreactor fermenting a honey solution with a specific gravity of 1.075. (●) Ethanol (% vol), (◆) Glucose (g/l), (■) Fructose (g/l), (▲) Sucrose (g/l), (X) Glycerol (% vol), (□) Immobilised biomass. The immobilised biomass represents a visual measurement of whether the biomass was in a packed bed (0-50 cm), fluidised (50-60 cm), or clear effluent with a few fluidised cells in suspension. The bioreactor inlet represents a height of 0cm and the outlet 117 cm.

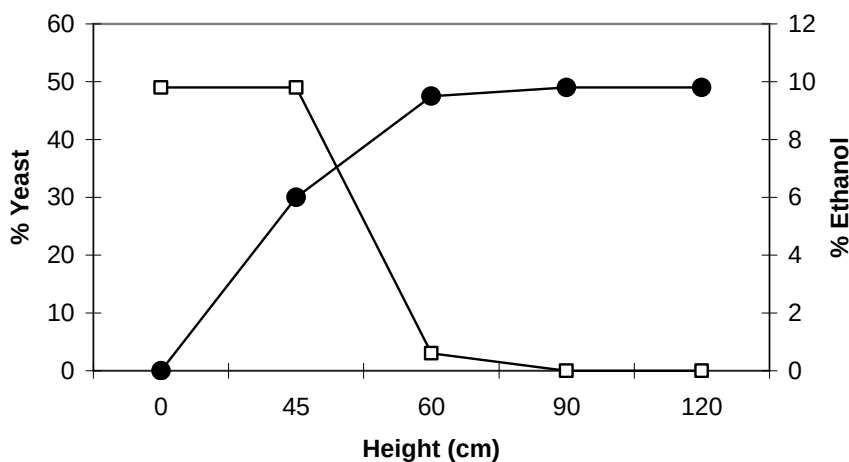


Figure 5.21 Data comparing the yeast bed (□), as described in Figure 5.19, to the percentage ethanol by volume (●), over the height of the column, with height 0 cm being the inlet and 117 cm the outlet.

Figure 5.22 shows the ethanol yield calculated as ethanol yield per gram of glucose or fructose. Sucrose converted to ethanol provides a slightly higher yield per gram due to the fact that the sucrose dimmer of glucose and fructose is 5% lighter than its separate constituents.

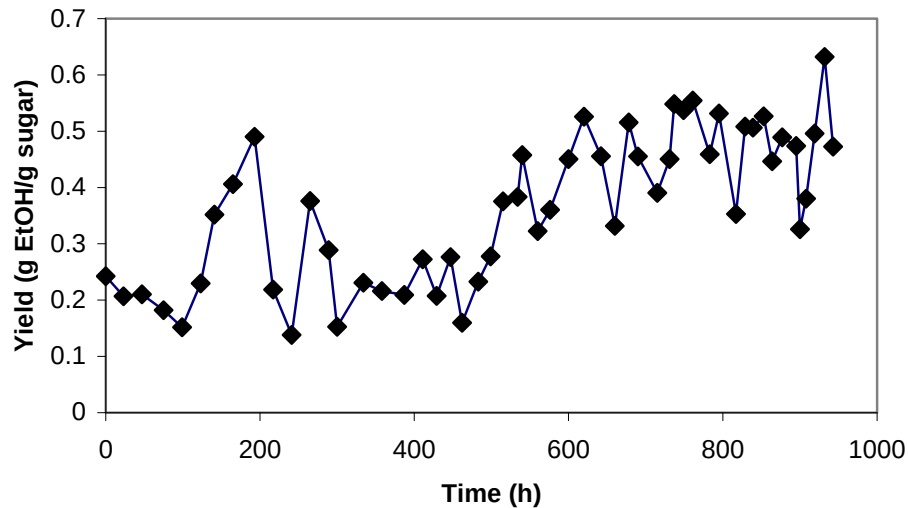


Figure 5. 22 This figure shows the ethanol yield in the bioreactor over time. The theoretical maximum yield is 0.51 g ethanol per gram of sugar (Dharmadhikari 1999). It is interesting to note that on certain occasions the ethanol yield was considerably above the theoretical maximum yield.

5.4.7 Packed bed characterisation

At 1000 hours of operation, the packed bed region of the glass tower bioreactor consisted of a 55 cm zone. This zone had a volume of 1 079 ml. Three days after feed from the pump had been stopped, the packed bed had settled into a solid, gas free plug with a volume of 824 ml. The drop in fluid level from the outlet, when the packed bed had become inactive, was taken to be the total gas hold up for the bioreactor and had a volume of 280 ml. The volume of root supports added filled a volume of 0.5 l.

These measurements allow the following deductions to be made:

If the inactive bed volume is subtracted from the active bed volume the non-solid volume of the packed bed is obtained with a volume of 255 ml. This volume would consist of a mixture of liquid and gas.

Although the total volume of the tower bioreactor is 3.3 l, the volume filled by liquid is obtained by subtracting the gas hold up volume and settled bed volume from the total, giving a liquid bioreactor volume of 2.196 l.

At a flow rate of 5.6 l/day, this gives a residence time of 9.4 hours.

In the packed bed region 128 g/l of glucose sugar equivalents were converted to 59.8 g/l of ethanol. This gives a yield coefficient of 0.47 which is 92.5% of the theoretical maximum yield of 0.51 grams ethanol per gram glucose consumed (Dharmadhikari 1999).

The total biomass in the packed bed region is the difference between the root support solid volume and the inactive packed bed volume. The yeast biomass had a volume of 409 ml. 1 ml of stationary phase strain F yeast had a mass of 1.16 g, hence a wet weight of approximately 474 g of yeast biomass was present in the packed bed.

Table 5.2 Ethanol productivity calculated for the point at which the profile was taken. Formula from Chen *et al.* (1994).

Head space volume	0.75 l
Liquid volume	3.30 l
Total volume	4.05 l
Flow rate per day	5.60 l
Final ethanol concentration	59.8 g/l
$\text{Volumetric Productivity} = \left(\frac{\text{EtOH (g/l)} \times \text{Flow Rate (l)}}{\text{Volume of Fermentor (l)}} \right) \times \frac{24}{24}$	
Volumetric Productivity	3.45g EtOH/l/hour

Volumetric¹ ethanol productivity using formula 5.2. This gives an ethanol productivity for the tower bioreactor of 3.45 g ethanol per l of bioreactor volume per hour (Table 5.2).

Given however that the ethanol concentration of the effluent did not vary after the first 1 079 ml of the bioreactor, this gives an ethanol productivity for the packed bed region of 12.93 g ethanol per litre of packed bed volume per hour.

¹ In this case volumetric refers to the volume of the bioreactor and not alcohol by volume

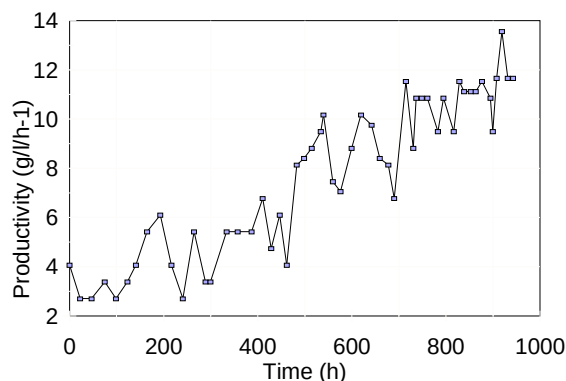


Figure 5. 23 Bioreactor bed productivity over time. An increase in productivity is evident, which is possibly attributable to strain acclimatisation in the bioreactor.

5.4.8 Organoleptic and alcohol analysis of aged samples

The product produced by the tower bioreactor was found to be acceptable to traditional *iQhilika* drinkers and instantly identifiable as *iQhilika*. The bouquet improved with age as did the smoothness of the product. Alcohol content by volume for matured samples was found to be 12% ($\pm .4\%$) on average. An experimental batch which was sweetened after tower fermentation reached an alcohol content of 17.2 % and had a pleasant sherry like flavour due to accidental oxidation. The deliberate oxidation of mead with the intention of creating a sherry like bouquet has been reported by Steinkraus & Morse (1966).

5.5 Discussion

The glass tower bioreactor with yeast strain F immobilised on 2 mm cubed *Trichodiadema intonsum* root supports was able to produce organoleptically acceptable *iQhilika* with a final alcohol volume of approximately 10% at a flow rate of 5.6 l/day.

The glass tower bioreactor performed as a reasonably stable, predictable system. The yeast immobilization support, *T. intonsum* roots, was able to retain as much as 474 g of yeast biomass in the packed bed. Despite the cubic nature of the root supports, biofilm covered the supports in a spherical layer. Bed expansion occurred

and was the result of gas hold up within the immobilised cell region. Flow rates were low enough that the terminal settling velocities of particles temporarily fluidised allowed them to be returned to the packed bed region – this correlated with data presented by Nicolella *et al.* (1999).

In the experiment in which strain F was grown on different concentrations of glucose, fructose and both (artificial honey) it was noted that the yeast flocculated very rapidly on fructose, whereas on glucose flocculation appeared to be more a symptom of the exhaustion of the glucose supply. In the honey mixture, flocculation of the yeast was at a later time than in the fructose, but at an earlier time than the glucose. This appears to be due to a diauxic growth pattern where the yeast rapidly utilizes glucose, flocculates, and begins utilizing fructose. Karasu-Yalcin & Ozbas (2004) reported that an osmophilic yeast, *S.cerevisiae* Narince 3, showed extremely low growth on fructose as compared to sucrose and glucose. Baldwin & Kubitschek (1984) showed that the buoyant density of *S.cerevisiae* cells varies during the cell cycle. The low growth rate on fructose may have resulted in a low buoyant density and hence increased tendency to flocculate. Soares *et al.* (1991) noted a direct correlation between moderate oxygen availability and high concentrations of glucose on flocculation. Given that the experiment conducted with strain F was under anaerobic conditions, it is possible that flocculation was delayed by low oxygen and high glucose concentrations. Berthels *et al.* (2004) reported a discrepancy between glucose and fructose utilisation in wine yeasts with certain yeast showing glucophilic tendencies. From the experiments in this chapter it is evident that that strain F is a glucophilic yeast.

Floc formation in yeast is a product of three phenomena, namely the formation of the flocs by particle collisions, the break-up of flocs as a result of erosion of particles from the surface and the splitting up of flocs (Van Hamersveldt *et al.* 1997). The concentration of cells at the base of a flask due to reductions in buoyancy cause increased likelihood of collisions and hence flocculation.

There is a direct correlation between yeast growth and reduced alcohol yield (Royston 1966). Royston noted that in situations where the correct conditions prevailed in a tower bioreactor, near zero cell growth rates could be achieved. These were linked to very high ethanol yields. Brown *et al.* (1981) showed that increases in

ethanol concentration in batch cultures inhibit growth more than fermentation. Oliviera *et al.* (1998) conducted a modelling analysis of the effect of ethanol concentration in a continuous tower bioreactor with a flocculating yeast and found that the model describing the relationship between ethanol concentration and production was linear (declining as concentration increased), whereas inhibition of growth of yeast was better described by a power law model.

In the *iQhilika* glass tower bioreactor initial ethanol yield was low, often half that of the theoretical maximum yield. In the initial stage of fermentation, a considerable portion of the sugars used by the yeast were converted to biomass. Some of this was retained in the bed and some washed from the packed bed region. Natural selection would have resulted in cells which were able to stay in the packed bed region being selected for, and hence a gradual increase in the cell density of the packed bed until a semi-stationary phase such as that seen by many operators of continuous bioreactors (e.g. Royston 1966, Chen *et al.* 1994) was obtained. At this point the primary metabolic activity of the yeast culture shifted from growth to maintenance. This is visible in the rapid increase in ethanol yields to the region surrounding the theoretical maximum yield of 0.51 grams of ethanol per gram of glucose or glucose equivalent. At a number of points during the fermentation the ethanol yield was considerably higher than the theoretical maximum yield. This is of course not directly physically possible as the production of ethanol follows a stoichiometrically balanced equation (Borzani & Jurkiewicz 1998). For comparative reasons the it is useful to compare the data in Figures 5.17, 5.18 & 5.19 with Figure 5.22.

The points of highest yield were almost always associated with an increase in glucose and fructose in the product. These sugars are primarily converted to ethanol during fermentation, hence it might be logical to assume that an increase in utilisation of sugars would lead to an increased ethanol yield, but the results are contrary to this. Bai *et al.* (2004) noted that in periods of extremely active fermentation yeast cells produce ethanol rapidly, which then causes cells to become less active as ethanol is toxic to the yeast. Dombek & Ingram (1986) suggested that yeast cells do not necessarily accumulate ethanol internally, but that internal and external ethanol concentrations are normally quite similar. Therefore it is possible that the yeast bed exhibits periods of very active ethanol production, followed by

periods of inactivity when ethanol rapidly diffuses from cells into the passing media. The decrease in activity would lead to an increase in substrate concentrations, which, in addition to the inhibition from the earlier high product concentration could also cause inhibition due to high substrate concentration – an effect which has been observed in continuous fermentations (Lenbury *et al.* 1999). Beuse *et al.* (1998) analysed the oscillatory behaviour of *S.cerevisiae* cells in continuous culture. They observed that at certain points in the cell cycle, yeast cells store carbohydrates in storage molecules and that at a later point they release the carbon in these molecules. Ethanol has been shown, together with variations in fermentable carbohydrate availability, to trigger cell resting and cell growth oscillations (Beuse *et al.* 1998). Other researchers have however shown that yeast carbohydrate storages is affected by glucose and ethanol concentration (Ogawa *et al.* 2000). Consequently two potential metabolic explanations exist for the higher than theoretically possible ethanol yield occasionally seen in the tower bioreactor, firstly that ethanol was being stored in cells and released during resting cycles, or secondly that during oscillations in cell growth and stored carbohydrate release, ethanol was produced from carbohydrates stored earlier. Borzani & Jurkiewicz (1998) in trying to explain yields of ethanol above the theoretical maximum concluded that certain other small undetected fermentable compounds result in this increase in yield – HPLC analysis of total sugars in the *iQhilika* must did not however show any other significant sugars present.

The possibility exists that sugars may be released from the root supports, but it is unlikely that the quantities would be large enough to influence the ethanol yields as the roots are not replenished with fresh roots, hence any sugars diffusing from the roots (which had a displacement of 0.5 l) could only have had a noticeable effect early in the operation of the bioreactor.

Honey is a highly soluble substance and the media in addition to being well mixed, was autoclaved prior to pumping into the bioreactor. It is possible however that occasional mixing irregularities may have caused irregularities in the feed into the bioreactor, although the probability of this is very low.

With time, the oscillations in yeast growth and ethanol production are likely to select for more and more ethanol tolerant yeast, resulting in higher and higher yields.

Brown & Oliver (1982) used a continuous selection system to produce ethanol tolerant yeast strains whereby the majority of the yeast was continuously inactivated by higher and higher ethanol concentrations, selecting for yeast which was not inactivated. A similar effect could have occurred in the tower bioreactor.

Glycerol production in the tower bioreactor increased with sugar concentration in the media. Nevoigt & Stahl (1997) showed that when yeast cells were placed in a hyper tonic solution, the response to the resulting efflux of water from the cell was to intracellularly accumulate compatible solutes which allowed enzymes and cellular components to continue functioning in the low intracellular water activities. This would explain the increase in general glycerol production seen as sugar concentrations in the media increased. The exposure of yeasts to high gravity sugar solutions is one of the ways that the metabolic balance can be shifted to favour glycerol production in commercial wine production and other fermentation systems designed to produce solutions rich in this heavy alcohol (Sahoo & Agarwal 2001, Wang *et al.* 2001, Balli *et al.* 2003).

The production of ethanol is a redox neutral process which consequently has no demand for NADH, but the production of glycerol consumes NADH (Wang *et al.* 2001). The packed bed region of the tower bioreactor is an anaerobic region. Ansell *et al.* (1997) detail how in anaerobic environments the synthesis of glycerol is crucial to the balancing of the cellular energy equation. It is likely that the synthesis of glycerol in the lower regions of the tower bioreactor, where sugar concentrations are highest, is an extremely important survival mechanism for the yeast. As the mead must (unfermented honey solution) progresses up the packed bed, the sugar concentration of the must declines as a result of yeast metabolism and the need for glycerol as an osmoprotective substance would decline. Its synthesis would however still be important to balance the cellular energy budgets of the yeast. When not needed intracellularly, glycerol is excreted both by export protein channels and by diffusion (Wang *et al.* 2001).

Analysis of media entering the tower bioreactor shows that slightly more glucose is present in the media than fructose. After passing through the immobilised yeast region this situation is reversed, suggesting the preferential fermentation of glucose. Berthels *et al.* (2004) found that elevated ethanol concentrations inhibited fructose

utilization more than it inhibited glucose utilization. The same authors however noted that at low ethanol concentrations glucose utilization was inhibited more than fructose utilization. In the fermentation results obtained for the glass tower bioreactor with Strain F immobilised on *T.intonsum* roots, a definite increase in the ratio of glucose to fructose in the effluent was evident at elevated ethanol productivities, suggesting that Strain F is a glucophilic yeast. Di Luccio *et al.* (2002), used an extreme version of a glucophilic yeast to produce ethanol from inverted sugar cane in such a way that the effluent would be rich in fructose which could then be isolated.

Shambaugh *et al.* (1990) noted that honey had a healthier effect on consumers than sucrose in terms of its impact on blood sugar. This was in part due to the fructose component, which is sweeter than sucrose. In the production of mead, the selective removal of glucose by the yeast and the higher levels of fructose in the final product may contribute sweetness without having a high actual residual sugar content. In the light of the trends towards low kilojoule health conscious products, this would be advantageous.

5.6 Conclusion

A tower bioreactor with *iQhilika* yeast strain F immobilised on 2 mm cubed *T.intonsum* root particles can be used to ferment a honey solution with an initial sugar content of 180 g/l to produce a mead with 9 – 10 % by volume ethanol and 10-12 g/l glycerol. Very high ethanol yields were achieved, resulting in efficient conversion of expensive honey sugars to ethanol. The *iQhilika* produced by the continuous bioreactor is acceptable to traditional brewers as *iQhilika* as well as being acceptable to many people not acquainted with traditional *iQhilika*.

If the technology can be scaled to a commercially viable scale, it would represent an efficient means for manufacturing *iQhilika* and mead in general. The rapid fermentation achieved through the use of immobilised *iQhilika* yeast and continuous fermentation is a substantial improvement on conventional mead fermentations.

The tower bioreactor produced a product containing approximately 9-10% alcohol by volume at a flow rate of 5.6 l/day. Within 3 days to a week of secondary fermentation in the maturation vessel this had increased to 12% alcohol. In contrast, batch mead

making systems reported in the literature take many weeks to 6 months. The shift from batch to continuous mead production represents one of the largest fermentation technological advances in the history of mead making.

Hence the objectives for this chapter have been achieved.

Chapter 6

Development of a commercial-scale *iQhilika* tower bioreactor

6.1 Introduction

In Chapter 5 it was shown that the traditional *iQhilika* bioreactor described in Chapter 2, a batch bioreactor with biomass immobilised on a support, could be adapted to operate as a continuous tower bioreactor, with the biomass/support forming a packed bed region with effective biomass retention. The tower bioreactor operated in Chapter 5 achieved high ethanol yields and produced mead with favourable organoleptic properties. The average final ethanol by volume of the mead produced, after maturation, was 12%. The bioreactor was operated for over 1000 hours without contamination.

Analysis of the profile of the bioreactor, performed by depth sampling across the packed bed region and the region above the support showed that across the height of the packed bed an even decline in the sugar concentration of the must, and an equivalently even increase in ethanol concentration occurred. This is indicative of a system with little mixing.

The bench-scale tower bioreactor operated satisfactorily as a system, but from an economic perspective its efficiency was low, with a daily production of 5.6 litres of mead. The process of taking data derived from a bioreactor of a specific size and using it to design a larger bioreactor is termed scale-up (Shuler & Kargi 1992). The primary objective of scale-up is to achieve better process economics (Atkinson & Mavituna 1991).

The production of mead at commercial scales is difficult due to the need for heating large volumes of must, which, due to scaling factors results in long heating times, long cooling times and denaturation of flavour compounds (Kime *et al.* 1991 a). Kime *et al.* (1991 a & b) tested a number of different methods to overcome this problem, such as flash heating in a tubular heat exchanger and ultra filtration. At any scale the problem remains that, in batches, mead ferments slowly, a factor which Steinkraus &

Morse (1966) overcame by addition of nutrients and growth factors and which Karuwanna *et al.* (1993) overcame by the addition of grape juice. The fermentation of mead has been hastened by agitation to improve mass transfer between the yeast and the must (Steinkraus & Morse 1966). Agitation of bioreactors is costly and involves careful design of the bioreactor vessel to avoid mass transfer problems (Ratledge & Kristiansen 2001).

From the perspective of scaling *iQhilika* production from bench to full-scale a number of these problems have been eliminated by the use of a continuous system. The primary problem in mead fermentation is that off-flavours will develop as a result of slow fermentation (Steinkraus & Morse 1966). The bench-scale continuous *iQhilika* bioreactor fermented the mead to an alcohol content of 8-9% by volume very rapidly. After this point the mead was resistant to contamination by spoilage organisms.

Continuous systems produce a constant stream of fermented product (Atkinson & Mavituna 1991). If a continuous bioreactor was designed to produce 330 l/day of mead, it would in the 4-6 weeks of a normal mead fermentation (Steinkraus & Morse 1966, Karuwanna *et al.* 1993) produce between 9 000 and 13 000 litres of mead. The continuous bioreactor would require a small heat exchanger or ultrafiltration unit to process the 13.5 litres of must needed per hour.

To sterilize the media for a batch process one could either use batch sterilization, heating the entire batch to the desired temperature and then cooling it, or a continuous system to fill a tank with processed media (Atkinson & Mavituna 1991). If a batch bioreactor is used and filled by a continuous process, a number of engineering and microbiological considerations have to be taken into account, such as presterilization of the bioreactor and filling of the bioreactor rapidly enough so that no contamination sets in.

Wiseman (1983) p.97 rephrases the famous philosophical tool, Occams Razor, to place it into a biotechnological context as, "systems should be no more complicated than really necessary". By using a continuous mead bioreactor such as that in Chapter 5 many of the scale up problems of batch mead fermentations are eliminated. The continuous tower bioreactor does not require mixing of the must as it

ferments, hence engineering problems concerning agitation are removed. The system operates continuously, hence media can be prepared continuously in small volumes. This eliminates many of the engineering problems associated with large-scale mead must preparation (e.g. Kime *et al.* 1991 a & b).

iQhilika production uses three wild derived products, honey, pollen and *Trichodiadema* roots. The production of these ingredients is an important factor in deciding the final scale of the bioreactor. The market for *iQhilika* produced using the continuous process would also have an impact on the optimal size of the bioreactor.

6.1.1 Honey supply

The supply of fermenting ingredients is an important factor in deciding the potential output of a bioreactor. The annual production of an average apiary consisting of 20 beehives is sufficient to produce 1000 litres of mead (based on data not reported here). The honey used in mead making contributes important organoleptic properties (Steinkraus & Morse 1966, Karuwanna *et al.* 1993). Although the objective of this chapter is to develop a commercial-scale continuous fermentation system, the product can be kept in individual containers with unique organoleptic properties derived from the honey used. Preparation of batches of must for fermentation would optimally be performed daily, hence the logical output of the bioreactor would divide into 1000 litres, the mead producible with the honey from one apiary. This would ensure organoleptic uniqueness to mead produced from honey from a specific area.

6.1.2 Availability of root supports

The *Trichodiadema intonsum* root supports used in the traditional fermentation are wild harvested. The root support requirement of the scale up experiments in this chapter is quite considerable and represents a harvest of approximately 800 plants. In the light of conservation concerns over wild harvesting it must be noted that *Trichodiadema* plants are specifically grown to provide root supports as is discussed further in Chapter 7.

6.1.3 Scale up strategies

Atkinson & Mavituna (1991) make the important point that scale up experimentation is expensive and it is important to perform adequate initial theoretical modelling of a process before pilot and commercial-scale systems are constructed. A bioreactor essentially provides an environment which is favourable to the production of the desired product (Wiseman 1983). The biological environment must be maintained in such a way that the desired organisms grow and no others (Wiseman 1983). The chemical environment must be maintained in a favourable state, which involves the maintenance of pH and mass transfer (provision of substrate, gasses and the removal of inhibitory substances) (Wiseman 1983). The physical environment, specifically temperature (Wiseman 1983) and pressure (Atkinson & Mavituna 1991), are important to biological cells.

In scaling a process up, these factors should change as little as possible to ensure that processes evaluated at laboratory-scale perform similarly at commercial-scale (Atkinson & Mavituna 1991, Shuler & Kargi 1992).

Scale up can make use of a number of different strategies:

Fundamental methods;

These methods involve the solving of microbalance equations for momentum, mass and heat transfer and are most applicable to homogenous systems and simple fluids (Atkinson & Mavituna 1991).

Semi-fundamental methods;

These involve the use of simplified equations, with a specific focus on flow and are applicable to plug flow, plug flow with dispersion and well mixed systems (Atkinson & Mavituna 1991).

Dimensional analysis;

This deals with the values of dimensionless groups of numbers, which are to be kept constant (Atkinson & Mavituna 1991). Dimensionless numbers such as the Reynolds number governing flow and the Nusselt number governing heat transfer are a few examples. Certain of these parameters are likely to be important in the scale up of

the *iQhilika* bioreactor. If for example flow rate past a support particle with immobilised biomass was higher in the final scaled bioreactor, the loss of biomass could be higher and the bioreactor may perform differently.

Rules of thumb;

Atkinson & Mavituna (1991) list certain rules of thumb which have been derived from observations of systems. These rules are essentially performance indices governing aspects of the bioreactor such as power per unit volume and torque per unit volume (Atkinson & Mavituna 1991). These may in some cases have applicability to another system.

Trial and error;

The gradual refinement of a system by testing and responding to negative outcomes with new attempts until such a time as a satisfactory outcome is achieved (Atkinson & Mavituna 1991).

Regime Analysis;

Atkinson & Mavituna (1991), define 'regime' in this context to mean the dominance of a mechanism in the functioning of a system and mention that a regime can be a pure one with only one mechanism controlling it, or mixed with multiple mechanisms influencing the system in comparable ways. Where dimensionless groups and scale-up criteria cannot be kept constant, regime analysis can determine which regimes are important to keep constant (Atkinson & Mavituna 1991). In this way, for instance in a bioreactor where oxygen mass transfer is determined to be a limiting factor, this regime would be dominant.

The data presented in Chapter 5 show that the continuous *iQhilika* tower bioreactor has a packed bed region in which the fermentation occurs, a clear section above that with a few fluidised root supports and adherent biomass and flocs, and a settler region above that, in which cells that are fluidised sufficiently vigorously to reach the top of the tower bioreactor lose momentum in the larger volume of liquid, swirl into side eddies, and eventually fall back down to the packed bed.

6.2 Objectives

To assess which scale up criteria are relevant to the continuous *iQhilika* bioreactor and use these to design a full-scale bioreactor.

6.3 Materials and methods

6.3.1 Determination of the size of the bioreactor

For practicality purposes it was decided that a bioreactor with capacity of 150 – 330 l/day would fulfil these requirements.

6.3.2 Root support preparation

Root supports were cut into 2mm² segments and placed in pressure vessels which were autoclaved at 120°C for 30 minutes. Root supports with a displacement of 500 ml were used in the bench-scale bioreactor (Chapter 5) which had a daily production of 5.6 l. To prepare a proportionally equivalent volume of roots for the full-scale bioreactor, segments with a displacement of 29 litres were prepared.

6.3.3 Selection of scale up criteria.

In Chapter 5 the bench-scale bioreactor was operated at a flow rate of 5.6 l/day. It had a liquid volume of 3.3 l with 0.5 l of root pieces immobilising 0.409 l of yeast biomass in the packed bed region. The gas and liquid volume of the yeast bed was 0.255 l. The gas hold up of the entire bioreactor was 0.280 l. The entire volume of the bioreactor, including headspace, was 4.05 l.

Analysis of the profile data across the packed bed region showed a regular decline in sugars and an increase in ethanol concentration (Chapter 5) until the end of the packed bed region, after which these values did not change much. The packed bed region exhibited predominantly non-mixed characteristics with extreme stratification across the bed.

The packed bed regions of continuous bioreactors can be described by a number of material balance equations (Shuler & Kargi 1992). In a yeast based fermentation system, yielding ethanol, in which cell growth is negligible (and consequently yield is close to the theoretical maximum) and diffusional limitations are negligible, the packed bed can be described by the following formula (Shuler & Kargi 1992):

$$S_{0i}-S_0=\frac{q_p X}{Y_{P/S}} \times \frac{A}{F} H \quad \text{(Formula 6.1)}$$

Where:

- S_{0i} = initial sugar concentration
- S_0 = final sugar concentration
- q_p = g ethanol per g dry cells per hour
- $Y_{P/S}$ = yield ethanol per gram of sugar
- A = cross sectional area in cm^2
- F = flow rate per hour in litres
- H = height of packed bed in meters

If this formula works for predicting the height of the packed bed region for the bench-scale bioreactor (Chapter 5) it can provide an indication of the dimensions of a packed bed for the flow rates required for the scaled up tower bioreactor.

Hence, the formula was solved for the bench-scale bioreactor and used to derive the dimensions of the packed bed region for a bioreactor with a maximum daily flow rate of 330 litres in a tower bioreactor with an internal diameter of 0.2 meters.

Some of the effects of the following parameters are not described by formula 6.1:

6.3.3.1 Flow rate

According to Kwok *et al.* (1998) biofilm formation is determined by the growth rate of immobilised cells and the detachment forces removing biomass. Shear, as defined by Atkinson & Mavituna (1991), refers to the movement of one layer in relation to an adjacent layer. *iQhilika* must be a moderately concentrated sugar solution. Ranjan *et al.* (2004) state that fruit juices, which have similar sugar concentrations to the *iQhilika* must, are non-Newtonian fluids. Sugar solutions are reported by Atkinson &

Mavituna (1991) to behave as Dilatant¹ fluids with viscosity increasing with shear rate.

In the case of a reasonably complete fermentation in the packed bed region, the must will have been converted from a Dilatant fluid at the base of the bioreactor to a near Newtonian fluid at the end of the packed bed. This would mean that in a packed bed one surface could be a biofilm coated support and the other surface could be the *iQhilika* must passing over it. As flow rate increased, so would shear, leading to detachment of biofilm and loss of biomass. Towards the end of the packed bed however the increase in shear proportional to flow rate would decrease where the must behaved more like a Newtonian fluid. The Reynolds number is defined by Atkinson & Mavituna (1991) as being the product of inertial forces divided by viscous forces. The dimensionless Reynolds number is useful in determining whether fluid flow will be laminar or turbulent within a packed bed containing either Newtonian or non-Newtonian fluids (Mishra *et al.* 1975). If flow through the packed bed is laminar or turbulent it will have an impact on mixing within the bed and on mass transfer as well as biomass stability. The break-up of yeast floc and biofilm is governed by two factors, namely the rate at which biofilm section and floc split, and the rate at which individual cells are eroded (van Hamersveldt *et al.* 1997). In certain cases high flow rates leading to high shear due to turbulent flow cause biomass to be split, detached, eroded and lost from the bioreactor leading to a decrease in bioreactor performance due to the loss of biocatalyst. Hsu & Wu (2002) modelled the effect of among other things shear on flocculated and immobilised yeast cells and showed that with reduced shear, they were able to maintain better biofilms than at higher rates of shear.

6.3.3.2 Gas evolution

In Chapter 5 it was evident that the bench-scale bioreactor rapidly converted sugars into primarily ethanol, glycerol, and carbon dioxide. The carbon dioxide left the packed bed region in the form of small bubbles which escaped the bed and rose to the surface of the bioreactor.

Ge & Li (2003) developed a powerful model to predict the effects of fluidisation of particles. A section of their model dealt with the effect of gas bubbles rising past

¹ A Dilatant fluid demonstrates an increase in apparent viscosity with increasing shear (Perry & Chilton 1982).

solid particles in a fluidised bed. The model showed that eruptions of gas could be predicted with resulting rapid movement of solid particles.

Eruptions were seen on a small scale in the bench-scale bioreactor (Chapter 5) and in terms of the operation of the scaled up tower bioreactor it would be important that these eruptions were not dangerously violent. To contain the eruptions and provide a buffer zone for rapidly released carbon dioxide, a large headspace volume would be useful. The gas hold up volume of the bench-scale bioreactor in Chapter 5 can be used as a starting point for calculating the average volume of gas that would be leaving the bed at any one point. For safety this could be multiplied by 10 and used to determine a safe head space volume to absorb pulses of gas. In the bioreactor design used in Chapter 5 both gas and liquid leave through the same exit. It is theoretically possible that an eruption of gas could fluidise sufficient root particles that the exit gauze would be blocked by these particles resulting in a need to unblock the exits which could introduce contamination. Hence designing a system to avoid this is desirable.

6.3.3.3 Time

In chapter 5 the bench-scale bioreactor was operated at a specific flow rate and hence the contact period between biomass and *iQhilika* must at a point in time would be constant. In order to scale up the packed bed region, the contact time between must and biofilm would have to be maintained at the same rate. If a constant contact time between biomass and *iQhilika* must were selected as an important regime in scaling up the tower bioreactor, the changes in gas volume with different tower heights and subsequent pressures would be important. Petrova *et al.* (2003), report that, in a column consisting of a packing material through which gas and liquid passes, when the column exceeds a certain height the distribution of gas changes dramatically. In the case of the mead fermentation, carbon dioxide is a product of fermentation and with increasing height and size of the packed bed region it is likely that these distributions of gas could lead to increased instability of the packed bed region.

Bearing these three sections in mind, the tower bioreactor can be designed. This would mean, in an extreme example, that the tower could not for instance be 100 m high and 3 cm wide as the flow rate past the root supports would be excessive and the gas hold up would become excessive due to the slow rising of gas through the

supports. The formula (6.1) could be used to predict the height of the packed bed region for a bioreactor with a flow rate of 330 l/day and a diameter of 3 cm, but these results would not be practical.

6.3.4 Stainless steel tower bioreactor construction

A grade 314 stainless steel bioreactor was constructed to the specifications determined by the above strategies for a 330l/day tower bioreactor.

6.3.5 Bioreactor operation

6.3.5.1 Sterilization

The bioreactor was filled with a 4% formaldehyde solution and left for three days, after which it was drained and left to stand for 2 hours, following which the tubular region and sides were rinsed with 180 litres of boiling water and drained. The liquid volume in the bioreactor was replaced with sterile filtered air.

6.3.5.2 Inoculation

The volume of root supports required was determined by dividing the volume of roots in the bench-scale tower *iQhilika* bioreactor by the flow rate and multiplying this by 330 litres. The root supports were placed in 20 litre pressure vessels and autoclaved for 45 minutes at 120°C. The area surrounding the manhole cover was liberally sprayed with 70% food grade alcohol. The pressure vessels were immediately removed from the autoclave, depressurised and the boiling contents (when the autoclave cools it cools to 100°C before the lid can be opened, but the pressure retained by the pressure vessel means that the temperature in the vessel is still above 100°C) were poured through the manhole cover in the top of the tower bioreactor.

Six 20 litre pressure vessels were filled with growth media for yeast biomass production as used in chapter 5. The vessels were autoclaved at 121°C for 45 minutes and allowed to cool. Pure colonies of strain F were inoculated into each vessel and sterile filtered air bubbled through the vessels for 4 days. The vessels were placed in a cool room to allow yeast to settle and the settled yeast pumped out into a sterile 5 litre flask. The settled yeast, with a volume of 6.3 litres, was

resuspended in fresh growth media and pumped into the base of the tower bioreactor.

6.3.5.3 Media and media sterilization

The same mead recipe as that detailed in Chapter 5 was followed.

Kime *et al.* (1991 a) used a heat exchanger to flash heat mead to 101 °C for half a minute followed by immediate cooling. To provide sterilized media for the full-scale tower bioreactor, a tubular heat exchanger was designed so as to be able to provide one and a half minutes of heat treatment at 100 °C followed by immediate cooling to 25 °C (Figure 6.1)

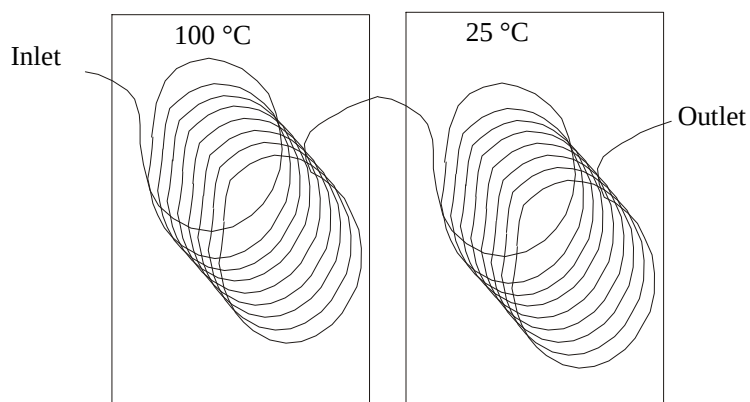


Figure 6. 1 The tubular heat exchanger design used consisting of a 6 meter coil of 316 grade 8 mm internal diameter stainless tubing submerged in a 15 litre volume boiling water bath. This coil was connected to a 3 meter coil in a room temperature water bath which was maintained at a constant temperature by a flow of fresh water into the bath (5 l/min).

6.3.5.4 Biomass development

Biomass immobilisation and growth was facilitated by pumping 1000 l of growth media as used in Chapter 5 to develop biomass on root supports. Initially media was pumped into the column in 20 l batches and allowed to ferment for 3 days prior to the addition of more media, until a total of 120 l had been added. Media was then pumped at a rate of 50 l/day for three days, and then 150 l/day for the remainder of the 1000 l.

The packed bed was allowed to go dormant by shutting the feed pump for three days. Once settled the uppermost sight glass was opened and a surface sterilised

pipe inserted through the sight glass port until just above the packed bed. A sterilised cotton bung was placed in the hole to make a tight seal around the pipe and the spent growth media was pumped out.

A honey solution with specific gravity 1.080 was pumped through the packed bed at a rate of 80 l/day initially and ramped to 150l/day.

6.3.5.5 Bioreactor operation

The bioreactor was operated for 1000 hours at a flow rate of 150l/day.

6.4 Results

6.4.1 Assessing the effectiveness of formula 6.1

The assessment of formula 6.1 (section 6.3.3) against results observed for the bench-scale bioreactor was performed using data in Table 6.1. The rate of sugar utilisation was determined from the profiles of the bench-scale tower bioreactor (Chapter 5).

Table 6. 1 Values used to solve formula 6.1 (section 6.3.3) and predicted bed height obtained. This corresponded with the 60 cm bed height observed in the operation of the bench-scale bioreactor under these conditions

Parameter	Value
q_p	0.204
X	65.96
$S_{0i}-S_0$	131.56
Diameter (cm)	5
A	19.64
$Y_{P/S}$	0.51
Flow rate per day (l)	5.6
Flow rate per hour (l)	0.23
Predicted Bed Height (m)	0.6

The accuracy of predictions with this formula suggests that the basic assumptions of negligible effect of diffusion of substrate into the biofilm and near total conversion of

substrate to product are validated. As a result of this observation it is not necessary to include the concept of a 'dead core', consisting of cells unable to perform due to diffusional limitations (Cruz *et al.* 2001) in the model.

To predict the dimensions of the packed bed for a full-scale tower bioreactor with a maximum flow rate of 330 l/day and an internal diameter of 20 cm, formula 6.1 was solved with the values in Table 6.2.

Table 6. 2 Values used to solve formula 6.1 (section 6.3.3) to determine the height of the packed bed in a full-scale *iQhilika* bioreactor.

Parameter	Value
q_p	0.204
X	65.95
$S_{0i}-S_0$	131.56
Diameter (cm)	20
A	314.29
$Y_{P/S}$	0.51
Flow rate per day (l)	330
Flow rate per hour (l)	13.75
Predicted bed height (m)	<u>2.2</u>

The predicted packed bed height was 2.2 meters

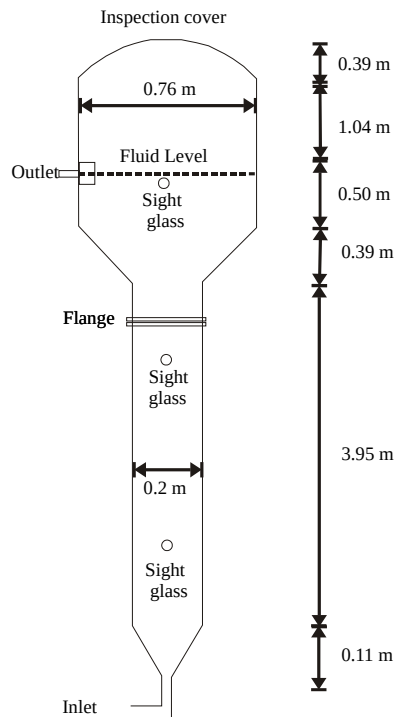


Figure 6. 2 The dimensions of the stainless steel tower bioreactor constructed to house the 2.2 m packed bed.

The full-scale tower bioreactor (Figure 6.2) was constructed with a level of conservatism. To maintain a constant ratio of packed bed depth to tubular region height this region would have been 3.3 meters – an additional 0.3 m safety margin was added.

The gas hold up in the bench-scale bioreactor was 0.280 litres. This would give the full-scale tower bioreactor an estimated gas hold up of approximately 16.5 litres. This multiplied by 10 for safety gives a required head space of 165 litres.

The difference in the cost of constructing the bioreactor with a head space of 165 litres and 480 litres was negligible. A bioreactor with 489 litres of head space, as measured after construction, was constructed.

6.4.2 Scale up construction

Figure 6.3 shows the scale up *in situ* at Makana Meadery.



Figure 6. 3 The grade 304 stainless steel tower bioreactor constructed according to the dimensions generated by the model.

6.4.3 Stainless steel tower bioreactor operation

The tower bioreactor (Figure 6.3) was operated at ever increasing flow rates until a constant attenuation was achieved. This flow rate was 150 litres per day.

6.4.4 Validation of predicted bioreactor characteristics

The scaled up tower bioreactor produced mead with a consistent flavour. The ethanol yield in the tower bioreactor was in the range of 0.4 grams ethanol per gram of sugar fermented (Figure 6.4). As with the bench-scale bioreactor in Chapter 5, it was noted that there were times when the *iQhilika* contained high concentrations of both unfermented sugars and ethanol giving rise to ethanol yields which are way above the theoretical maximum of 0.51 grams ethanol per gram sugar (e.g. Dharmadhikari 1999).

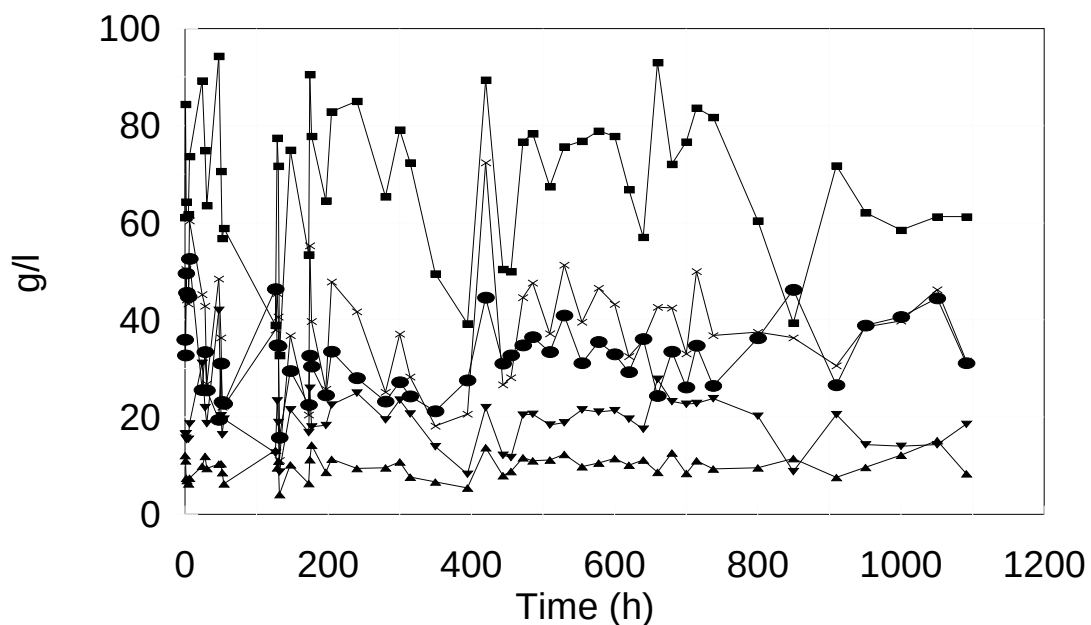


Figure 6. 4 Operation parameters (Specific Gravity of must and flow rate) and measured *iQhilika* characteristics for 1000 hours of operation. (●) Ethanol (% vol.), (▼) Glucose(g/l), (■) Fructose(g/l), (▲) Sucrose(g/l). The Ethanol yield (g EtOH / g sugar) has been multiplied by ten and placed on the same axes (X). The values for sugars are for the residual sugar in the effluent. Samples of influent were taken and used for calculations later in this chapter, however influent sugar concentrations are not shown here.

6.4.5 Full-scale tower bioreactor productivity

The volumetric productivity of the full-scale tower bioreactor was calculated by multiplying the ethanol concentration in the effluent by the daily flow rate then dividing by the volume of the bioreactor and then by 24 to give ethanol productivity in grams per litre per hour.

Ethanol productivity (Table 6.3) for the packed bed region for the last 200 hours was calculated using formula 5.2 (Chapter 5, section 5.3.9)

Table 6. 3 Ethanol productivity calculated for the average of the last 200 hours of operation. Volumetric productivity formula from Chen *et al.* (1994).

Head space volume (l)	489
Liquid volume (l)	735
Total volume (l)	1224
Flow rate per day (l)	150
Final ethanol concentration (g/l)	37.8
$\text{Volumetric Productivity} = \left(\frac{\text{EtOH (g/l)} \times \text{Flow Rate (l)}}{\text{Volume of Fermentor (l)}} \right) \div 24$	
Volumetric productivity (g EtOH/l/hour)	0.19

To compare the productivity of the packed bed region of the full-scale tower bioreactor to that of the bench-scale bioreactor (Chapter 5), the productivity was calculated specifically for the 69 l section which contained the packed bed region (Figure 6.5). For comparative purposes Figure 6.6 shows the equivalent data for the bench-scale bioreactor.

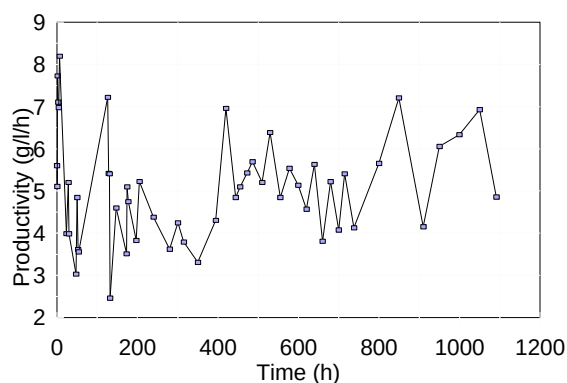


Figure 6. 5 Productivity for the active bed region of the scaled-up tower bioreactor over time.

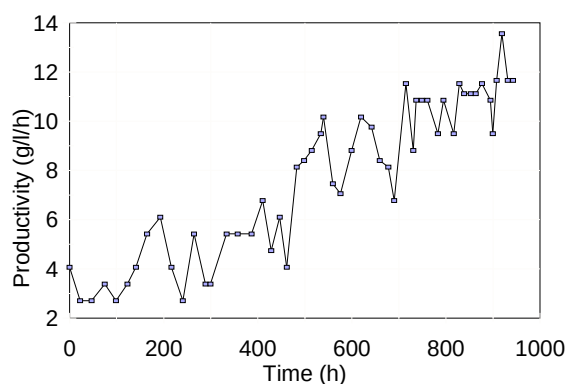


Figure 6. 6 Productivity of the packed bed region for the bench-scale bioreactor over time.

It is evident that ethanol productivity was higher in the bench-scale bioreactor than in the scale-up tower bioreactor. The ethanol productivity in the bench-scale bioreactor gradually increased with time whereas the increase was less noticeable in the full-scale tower bioreactor.

The traditional bioreactor (Chapter 2) had a volumetric productivity of 0.74 g EtOH//hour. The productivity of the liquid region of the bucket bioreactor was 0.8 g EtOH//h. The yields and productivities for the full-scale tower bioreactor were considerably higher than those for the bucket bioreactor, but were lower than those for the bench-scale bioreactor. Table 6.4 compares the productivity of the packed bed regions of the two tower bioreactors and the traditional bioreactor. The biomass in the traditional bioreactor was dispersed throughout the volume of the bioreactor.

Table 6. 4 A comparison of the productivity of the biomass containing sections of the three bioreactors used in this thesis.

Bioreactor	Bed Productivity
Traditional	0.8 g EtOH//h
Bench-scale (average for last 200hrs)	12.9g EtOH//h
Full-Scale Tower (average for last 200hrs)	3.4g EtOH//h

6.4.6 Organoleptic analysis

Tasting of matured *iQhilika* revealed greater consistency in flavour than that obtained with the bench-scale bioreactor. The product still retained the essential *iQhilika* characteristics, but was softer and smoother in general taste.

6.5 Discussion

The general performance of the full-scale tower bioreactor was lower than that of the bench-scale bioreactor. Productivity per unit active volume was considerably lower. Many authors report that full-scale bioreactors frequently operate at below the efficiency of pilot-scale lab bioreactors (e.g. Atkinson & Mavituna 1991, Shuler & Kargi 1992, Hsu & Wu 2002).

Although unit productivity of ethanol decreased in the scaled up tower bioreactor, the organoleptic qualities of the product improved. Hence a trade off between ethanol productivity and product quality occurred. In the light of this improvement in product quality, the slight decrease in productivity is not considered a serious operational concern.

Different types of honey ferment at different rates (Steinkraus & Morse 1966). This variability can be reduced by the addition of growth factors and nutrients (Steinkraus & Morse 1966). The honey used for the bench-scale tower bioreactor was a regional wild flower honey produced by Makana Meadery. The honey used for the testing of the full-scale commercial tower bioreactor was a purchased blend (Fleures, Pretoria) consisting of sunflower and citrus from South Africa, Argentina and China. Although these honey samples were very similar in their sugar composition, it may be possible that some other factor in the wild flower honey encouraged fermentation, or that a factor in the purchased blend decreased the rate of fermentation.

6.6 Conclusion

With the selection of appropriate scale up criteria and strategies it was possible to produce a 1:59 scale continuous bioreactor capable of producing 150 l/day of

iQhilika in a similar way as the bench-scale 5.6l /day bioreactor. Although a decline in productivity occurred, the organoleptic properties of the product improved.

The objective of designing a system which exactly duplicated the operation of the bench-scale reactor at a commercial-scale was not entirely met, but the scale up operated in a sufficiently similar way, and in some cases a better way, than the bench-scale reactor. In this light, the scale up was a success.

The commercial operation of a continuous mead producing bioreactor at flow rates of 150 l/day represents a significant advancement in the commercial production of mead. It demonstrates that the new technology discussed in Chapter 5 can be commercially implemented. When compared to other commercial mead producing systems which take weeks to months to produce product, this represents a significant biotechnological advancement.

Chapter 7

Issues affecting the development of *iQhilika* production

“...let us not forget that the African continent is immense, not only in terms of its size but, more importantly, with respect to the cultural, linguistic, and ethnic diversity that characterises the people who live in its various parts. Its biggest challenge is poverty and underdevelopment.

In this regard, higher education in Africa is faced with considerable challenges related to the eradication of poverty. These include the development of our human resources and the promotion of indigenous knowledge systems. “

South African President Mr Thabo Mbeki 2005

7.1 General Introduction

The objective of the research presented here was to develop a technology platform to produce *iQhilika* which would meet legal requirements in terms of South African law, and which could be marketed through commercial liquor distribution channels to supply markets which currently no longer have access to mead. As was discussed in Chapter 1 and 2, the traditional beverage, produced using IKS of the region, is currently proscribed by the authorities and with time faces extinction due to this pressure. On the other hand, population growth and the associated proportional increase in demand for ingredients for *iQhilika* production, has made traditional production of the beverage unsustainable and could result in much the same effect.

While the technology platform developed was shown to produce mead efficiently, a number of other issues were considered necessary to allow the work to be successfully commercialised.

The commercialisation of *iQhilika* using the industrial-scale continuous tower bioreactor developed in this study, and additional stimulation of the traditional mead making industry, both objectives of this programme, would create a substantially increased demand for honey, pollen and *T.intonsum* roots. In order to be environmentally sustainable, systems for the production of these ingredients would have to be developed.

The wild harvesting of *Trichodiadema* roots and bee products from fragile ecosystems is unsustainable. In the Eastern Cape a large population of very poor people live in these fragile ecosystems and in many areas this has resulted in the near total destruction of wild honeybee and *Trichodiadema* populations. The industrialisation of *iQhilika* has the potential to draw money from these rural economies, as is the case with other commercial beverages. The production of the raw materials for *iQhilika* production is, however, possible in agriculturally marginal regions in the Eastern Cape where the ingredients for other beverages cannot be produced. Hence establishment of an *iQhilika* industry has the potential to reverse the flow of money from these rural areas to industrialised urban areas, through the development of beekeeping and *T.intonsum* farming enterprises.

The initiative to undertake the commercialisation of *iQhilika* should thus comply with the best models of triple bottom line accounting including firm economic, social and environmental bottom lines.

In the year 2000, Makana Meadery was founded in partnership with Rhodes University, Grahamstown, to commercialise *iQhilika*, with the primary objective of effective technology transfer and beekeeping development so as to maximise the positive impact on rural economic development.

Among the issues addressed, the following will be discussed in this Chapter:

- The development of a sustainable supply of *Trichodiadema intonsum* roots.
- The development of a sustainable supply of pollen
- A model of *iQhilika* commercialisation as a rural economic enterprise

Although the development of honey production in South Africa was researched, it has not been dealt with here as this topic has been well reviewed elsewhere (May 1961, May & Tromp 1969, Institute of Natural Resources 2003).

7.2 Cultivation of *Trichodiadema intonsum* for root support provision

7.2.1 Introduction

The wild harvesting of plant products has had considerable negative environmental effects, with a number of South African indigenous species becoming very rare (Zschocke *et al.* 2000, Pfab & Scholes 2004).

Fennell *et al.* (2004) stress that in commercial cultivation of traditional plants the medicinal properties of the plant may change. In the case of *T. intonsum* in *iQhilika* production the plants appear to be required for the roots to immobilise yeast.

In order to assess the cultivation of *T. intonsum* it would be necessary to assess the growth rate and the efficiency of cultivated roots in fermentation.

7.2.2 Objectives

The cultivation of *T. intonsum* was investigated to assess if the plant can be produced sustainably under commercial crop production conditions and if the root supports produced remain effective in *iQhilika* production.

7.2.3 Materials and Methods

7.2.3.1 *Trichodiadema intonsum* cultivation

Obtaining sample plants

The Selmar Schonland Herbarium, housed at the Albany Museum, Grahamstown, was visited and a specimen of *T.intonsum* which had been harvested from the Ecce Pass, a dry rocky mountainous region near Grahamstown, was found. The sample site was visited and a large population consisting of over 400 plants was discovered. Ten plants were uprooted and transplanted to plastic plant pots filled with soil consisting of 2 parts clay, 1 part compost and 1 part vermiculite.

Obtaining Seeds

The potted plants were placed in the open and observed to flower after rains in spring and summer. The seeds were harvested and planted into seed trays.

Once the seedlings had filled the seedling tray wells they were transplanted to open ground in direct sunlight. The soil was improved by the addition of 5 l of milled composted pine bark per square meter.

Vegetative cuttings

2cm, 4cm and 6 cm sections of stem were cut and placed in seedling trays with the stems embedded a minimum of 2cm into the soil.

Once established, cuttings were transplanted to open ground in direct sunlight in soil which had been cultivated and improved by the addition of 5 l of milled composted pine bark per square meter.

7.2.3.2 Assessment of the effectiveness of cultivation

Plants which had been grown for 6 months and for 1 year were uprooted. The soil was washed from the roots of the plants. The volumetric displacement of the roots were measured by placing them in an Archimedean displacement vessel to determine volumetric growth of the roots.

7.2.3.3 Assessment of the acceptability of cultivated roots to traditional brewers

Two brewers, Mr Makwana and Ms Zabo were provided with peeled cleaned roots derived from cultivated plants. The source was not mentioned but they were asked to evaluate the roots in *iQhilika* production and comment on customer reaction to the batches so prepared.

7.2.4 Results

7.2.4.1 Seedlings and vegetative cuttings

Both seedlings and plants derived from vegetative cuttings produced healthy plants. Seedlings took 50 days to reach a transplantable state and cuttings took 30 days to reach the same stage.

7.2.4.2 Growth Rates of *T. intonsum* seedlings

After six months of growth, plants showed little overall variation in size, whereas after a year considerably greater variation was evident (Figure 7.1).

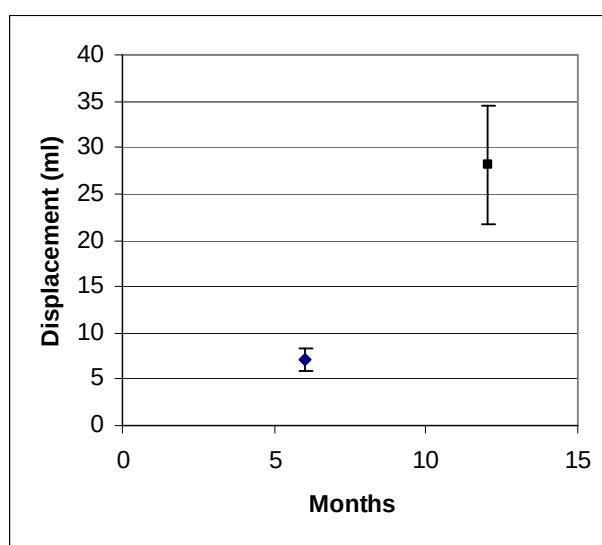


Figure 7. 1 This graph shows the volumetric displacement for samples of 6 plants of 6 months old and one year old

After 6 months of growth the plants had all produced three harvestable root segments (Figure 7.2). A number of seedlings were found growing under the plants but none had produced storage roots yet.



Figure 7. 2 The reasonably uniform growth of plants at 6 months is evident.

After a year of growth (Figure 7.3) the primary plants had entirely covered the surface surrounding them and in some cases where branches touched the ground, secondary plants developed (Figure 7.4).



Figure 7. 3 After a year of growth the uniformity between plants was less evident. Certain plants invested more in aerial growth, whereas others produced clusters of finely interwoven roots. All plants were however harvestable.



Figure 7. 4 Where branches of *T.intonsum* contacted the soil, new plants developed and these in turn rapidly developed into harvestable roots.

Trials in open soil proved successful with the *T. intonsum* plants rapidly covering the ground surface and propagating themselves vegetatively (Figure 7.5).



Figure 7. 5 A patch of *T. intonsum* in pine bark improved soil. The first half of the bed, closest to the viewer consists of seed derived plants and the second half of cutting derived plants.

7.2.4.3 Acceptability of cultivated roots to traditional brewers

The roots provided to Mr Makwana and Ms Zabo performed satisfactorily and produced marketable *iQhilika*. Both brewers expressed satisfaction with the roots and were not discouraged to hear that the roots were from cultivated plants. Ms Zabo requested to purchase additional roots.

7.2.4.4 Pests and problems

No major problem with pests was observed. Slugs occasionally ate seedlings leaves, but the plants recovered. Over-watering is the single largest cause of plant mortality – a problem which is remedied by the addition of pine bark chips and/or vermiculite to the soil. The plants were observed to be frost tolerant and tolerated extremes of heat. The plants grew faster as an understory filling in the spaces below larger plants which provide protection from the elements. De Villiers *et al.* (2001) observed that in the succulent Karoo ecosystem, the growth of many species in an understory is beneficial and enhances the stability of the ecosystem. *Trichodiadema* plants growing in the wild were observed to grow in this fashion.

7.2.5 Discussion

The cultivation of *T. intonsum* is possible with plants reaching a harvestable size after 6 months and quadrupling in size after another 6 months. The roots were accepted by traditional brewers. *Geisho*, the plant ingredient added to *tej* is produced by subsistence farmers in rural Tigray, Ethiopia, for sale and represents a dependable source of income for rural people (Vetter 1997). It is possible that the production of *T.intonsum* for *imoela* preparation could represent an income source for rural people in the Eastern Cape.

The roots of the *T.intonsum* plants were observed to be harvested and the stems and leaves were normally discarded by traditional gatherers. In the interests of sustainability the stems can be replanted, and with each harvest a greater number of plants can be produced.

7.2.6 Conclusion

The results of this study have shown that *T.intonsum* can be successfully cultivated and the roots produced in this way used in the effective production of *iQhilika* by traditional brewers. This finding provides an opportunity to relieve pressure on the fragile Nama Karoo biome where large quantities of *T.intonsum* plants are harvested. It also provides a potential source of income for rural farmers who can cultivate this plant for sale in urban areas. From the perspective of the commercialisation of *iQhilika* production at Makana Meadery it meant that this could be pursued without negative impact on *T.intonsum* populations.

7.3 Provision of pollen for *iQhilika* making

7.3.1 Introduction

Honey gathering from wild beehives has a destructive effect on these hives and causes huge numbers of beehive colonies in Africa to perish (Hepburn & Radloff 1998). Hepburn & Radloff (1998) place the number of beehives destroyed by man in Africa per annum in the region of 15 000 000 colonies.

Theft of beehives has been the largest factor causing the slow growth of the South African beekeeping industry (Institute of Natural Resources 2003). Discussions with 13 commercial beekeepers showed that traditional *iQhilika* production represents the single largest threat to beekeeping in the Eastern Cape with beehives being raided for brewing ingredients. Pollen is used by traditional brewers, with or without brood, to invigorate the fermentation.

In traditional *iQhilika* production, harvesting of the beehive components has a negative environmental impact. In order to sustainably produce *iQhilika*, it is necessary to ensure that pollen can be produced on a large scale to limit the wild harvesting and theft of pollen for *iQhilika* production

Honeybees have been commercially farmed throughout Africa (Hepburn & Radloff 1998) and the transition from honey gathering to honey farming is facilitated through technology transfer.

In traditional African beekeeping, beehives are kept in various types of enclosures, ranging from hollow trees, bark sections, pots, wattle and daub boxes, to straw baskets (Clauss & Clauss 1991, Ntenga & Mugongo 1991, Forestry Department Report 1992). The bees are allowed to fill a container and the honey is removed by opening the container and cutting combs out, aided by copious smoke to calm the bees. The honey harvesting often leads to the destruction of the hive, or serious damage thereof (Hepburn & Radloff 1998). The invention of the Langstroth hive (by Reverend Langstroth) revolutionised beekeeping in much of the world as it allowed the combs within a hive to be efficiently manipulated, and honey extraction did not require the destruction of the colony (Root *et al.* 1975). For various reasons this hive has met with limited acceptance in Africa, and the closest widely used hive with moveable frames is modelled on the Kenyan top bar hive (Clauss 1983, Ntenga & Mugongo 1991). This hive also allows movement of frames but lacks much of the strength of the Langstroth hive.

Beekeeping in South Africa has not been a traditional occupation and has largely been carried out by commercial beekeepers who employ the western Langstroth beehive (May 1961, May & Tromp 1969). Recent problems, such as the invasion of the parasitic mite of the honeybee, *Varroa destructor*, have influenced honey production, but natural selection has resulted in resistance to the mite developing and this is no longer as significant a threat (Dr Mike Alsopp¹ pers. com.).

Pollen can be sustainably trapped from a beehive using a pollen trap (May 1961, May & Tromp 1969, Root *et al.* 1975). The honeybee gathers pollen on its pollen basket or corbicula (Flower 1952). When the bee returns to the hive it passes through a screen which allows the bee through but dislodges the pollen from the corbicula, with the pollen then falling into a basket below the hive (Roodt *et al.* 1975).

In the Eastern Cape a wide range of flowering plants are found. In a study conducted at a site half a kilometre from an apiary used for providing honey for research in this thesis, 97 families of flowering plants, 271 genera, and 448 species of vascular plants were identified (Admassu Addi 2003). The plants providing large volumes of pollen were however fewer in number.

¹ Dr Allsop is a honeybee researcher at the Plant Protection Research Unit of the Agricultural Research Council in Stellenbosch and has observed the effects of *Varroa destructor* on *Apis mellifera capensis*.

7.3.2 Materials and methods

7.3.2.1 Pollen trapping

Two pollen traps modelled on the Farrier trap (Roodt *et al.* 1975) were obtained. Two beehives were used to trap pollen in June 2002 (*Eucalyptus saligna* flowering season) and in October 2004 (spring wildflowers) at Makana Meadery. The vegetation in the area consisted of 12 hectares of *Eucalyptus saligna* forest and a mixture of mountain fynbos, weed-covered fire damaged land and wetlands. Many different annual plants flower in spring and produce a mixed pollen flow.

Pollen was harvested once a week and weighed.

7.3.3 Results

7.3.3.1 Pollen trapping

The pollen flow from *E.saligna* is very strong but due to the short photoperiod and cold weather conditions in mid winter the bees only fly from 11.00 am until 3.00 pm on sunny days. The cold frontal systems which frequent the region in mid-winter further suppress flying activity, resulting in the low pollen recovery seen in mid June (Figure 7.6) despite this being at the peak flowering of *E.saligna*.

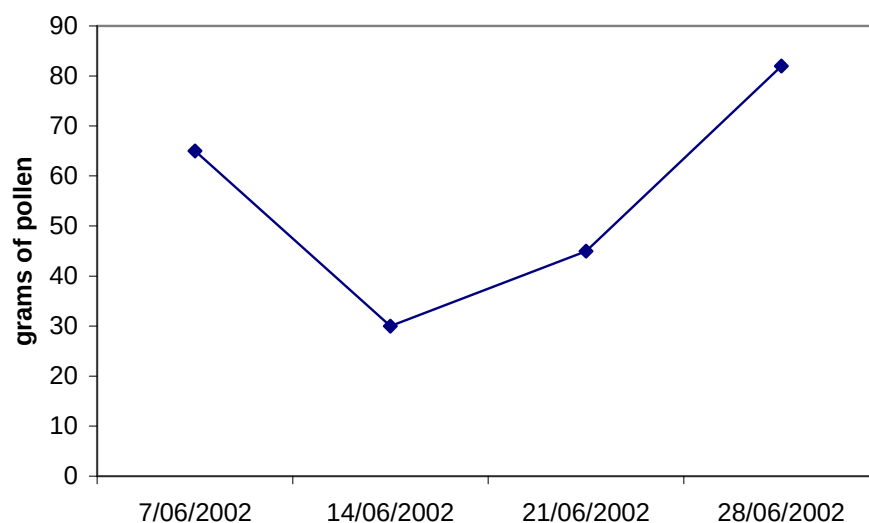


Figure 7. 6 Pollen yields from a single storey hive at Makana Meadery in mid-winter during the *Eucalyptus saligna* honey and pollen flow.

In spring, flying conditions were considerably improved and bees flew from 7.00 am until 5.00 pm on most days. This resulted in a higher pollen yield (Figure 7.7). The pollen obtained was entirely mixed in colour and pellet size.

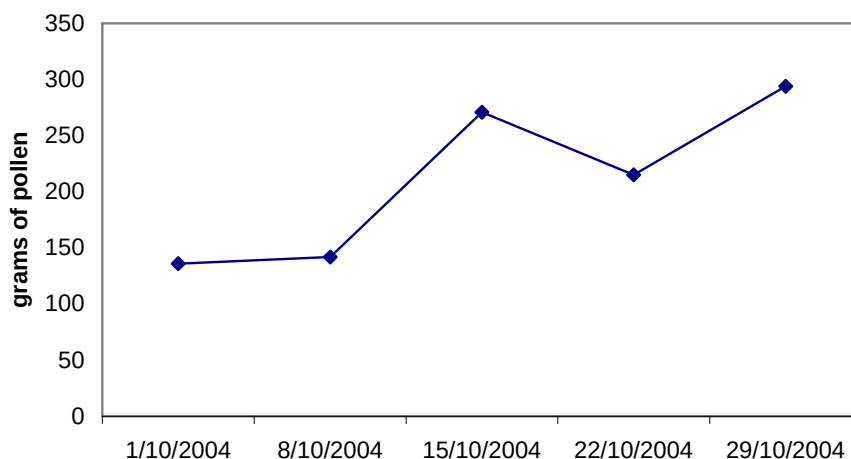


Figure 7. 7 Pollen yields from a single story hive in spring. This pollen is multi-floral.

7.3.3.2 Pollen testing in *iQhilika* production

The pollen of *E.saligna* was acceptable and invigorated the fermentations both of the traditional trial brewers, Mr Makwana and Ms Zabo. Both brewers, however, noted that the smell of the pollen was unpleasant and made the *iQhilika* taste strange. This suggests that although a good nutrient source for yeasts, some aspect of the utilisation of pollen from Eucalypts releases malodorous breakdown products. This smell has also been noted in beehives foraging on *E.saligna*.

The wild flower pollen was favourably received and it produced highly acceptable *iQhilika* batches. Mr Makwana noted that when he brewed with pollen he was able to produce *iQhilika* for longer before having to dry his root preparation to reduce sourness.

Ms Zabo has since purchased pollen at R 10 per 250 ml from Makana Meadery for brewing purposes. Two other producers, one on a farm, and one in town occasionally buy pollen as well. Despite demonstrating how to trap pollen, the

technology has not taken root among these brewers who prefer to purchase the product.

The primary reason cited by brewers wishing to purchase pollen is that they find it produces *iQhilika* which is of a higher quality than if brood is used and that the *iQhilika* does not sour as fast.

7.3.4 Conclusion

Pollen can be trapped and used to invigorate the root biomass (*imoela*) preparation. Given the demonstration of acceptability of pollen to traditional brewers, and their willingness to purchase it rather than products obtained from hive raiding, this indicates that another aspect of *iQhilika* production can be sustainably produced.

7.4 Commercialisation of iQhilika as a rural economic enterprise

7.4.1 Introduction

The production of *iQhilika* using the scaled up tower bioreactor at Makana Meadery is not in itself a labour intensive process and hence does not have a major impact on the community in terms of employment creation. Beekeeping, and the provision of beekeeping services do have the potential to create employment opportunities.

Beekeeping requires beehives, which can be made from wood. The South African Department for Water Affairs launched the Working for Water (W4W) programme in 1995 to control the spread of alien invasive vegetation. The programme has in addition to the control of alien vegetation types which consume water, targeted the creation of employment opportunities. The long term outcome of alien vegetation clearance has been improved water security and workforce development.

Within the Grahamstown region, the W4W programme dealt with encroachment of predominantly pine, eucalypt and wattle species. The trees are typically not large, and have many branches. Beehives are constructed from small pieces of timber.

The W4W programme clears timber that is not of commercial value but can be turned into beehives.

The construction of beehive components and beekeeping are both labour intensive occupations.

7.4.2 Objectives

To commercialise *iQhilika* in such a way that it maximised job creation

7.4.3 Materials and methods

7.4.3.1 Factory Design

The *iQhilika* production system was designed to minimise mechanisation in the following way:

Mixing of mead – mixed using a paddle by hand

Bottling of mead – hand operated bottle unit

Corking of mead – hand operated corker unit

Sealing of bottles – hand operated wax sealer

7.4.3.2 Beekeeping support products

Beekeeping equipment was provided by:

- Setting up a sawmill for lumber processing

- Setting up a workshop for timber processing

- Setting up a wax press for comb foundation making

Beekeeping services were provided by:

- Establishing a beekeeping training course

- Producing beekeeping manuals and a video for the Eastern Cape

- Training an extension team to provide follow up support for trainees

7.4.3.3 Beekeeping

Beekeeping was divided into two sections:

Commercial Beekeeping Unit

The objective of this unit was to establish a migratory beekeeping operation producing high average honey yields per hive.

Beekeeping Development Unit

The objective of this unit was to establish community projects with small scale honey producers and transfer beekeeping technology thus creating employment opportunities.

Commercial Beekeeping Unit

This unit was established and modelled on the North American migratory beekeeping system. Two Makana Meadery beekeepers worked on an exchange programme in Canada with a large beekeeping firm to learn commercial beekeeping systems.

This foundation was used to set up experiments testing migratory beekeeping in the Eastern Cape.

The commercial unit also acquired beehives by trapping swarms and removing problem swarms from houses in urban areas.

Beekeeping Development Unit

This unit operated by sourcing funding for a group of beekeepers to be trained and then providing training and beekeeping equipment to others. Follow up beekeeping extension services and honey marketing was also provided.

7.4.4 Results

7.4.3.1 Factory Design

The labour intensive production system designed can produce 50 bottles per hour and required 2 - 4 people to operate. It had a maximum daily output of 400 bottles per day. Figure 7.8 shows the production line.



Figure 7.8 Bottling Makana Meadery's first big order of 1500 bottles (January 2004). The picture on the right shows the simple yet effective wax sealing process.

7.4.3.2 Beekeeping support products

The sawmill required a logging team of four people and a lumber processing team of three people (Figure 7.9).



Figure 7.9 The sawmill converted low quality logs as seen on the left into timber using a portable Petersen sawmill unit seen in operation on the right. The process was labour intensive.

Timber processing to beehives (Figure 7.10) required six people to produce ten complete assembled and painted beehives per day. Wax processing required one person to produce 100 sheets per day. 100 wax sheets were adequate to place a 1/3 wax sheet in each frame in the ten beehives produced per day.



Figure 7. 10 The manufacture of beehives is a labour intensive process.

The beekeeping support programme required two people for three days of each month.

7.4.3.3 Beekeeping

Commercial Unit

The commercial unit performed the beekeeping experiments detailed in section 7.4. In addition the unit trapped 137 swarms between 2001 and 2005, performed in excess of 150 bee removals and purchased 50 beehives. The problems with the parasitic mite, *Varroa destructor* resulted in the Makana Meadery hive count ranging from over 200 hives in early 2002 to 58 hives in late 2003. The evolution of resistance to *Varroa* in late 2003 and the increased presence of wild swarms saw beehive numbers increase to close to the 200 mark by late 2004. The commercial unit required three people to operate.

The commercial unit began to implement advanced beekeeping practises learnt through international exchanges to Canada (Figure 7.11).



Figure 7. 11 Makana Meadery founding director Mr Vuyani Ntantiso and Mr Phumlani Honi working in a large beekeeping operation in Canada (left) and visiting a commercial beekeeping and mead making concern, Munro's Meadery (Right). This allowed Mr Ntantiso to explain the competitiveness of international beekeeping to trainees in South Africa.

Beekeeping Development Unit

This unit trained 19 small scale beekeepers who had never kept bees before. These beekeepers averaged 12 kg of honey per hive (Figure 7.12).



Figure 7. 12 A group of trainee beekeepers work their apiary near Port Elizabeth. The apiary is sited in valley thicket vegetation and produces excellent honey.

The unit assisted 14 existing beekeepers by providing additional training, equipment and beekeeping consumables.

7.4.4 Discussion

The design of an entire system to produce maximal employment has resulted in the creation of a number of employment opportunities. In many cases due to work schedules that are not completely filled in one portfolio, staff at Makana Meadery move from one portfolio to another. Under optimum conditions however, each section of work has been designed in such a way that it can employ one person entirely. The peak employment of Makana Meadery from 2003 - 2004 was 16 individuals during the establishment phase.

In the South African context this is an important example to set as many businesses are opting to mechanise and downsize work forces. In the case of a small town such as Grahamstown, designing a factory to maximise employment retains income in the town, contributing to growth and development.

7.4.5 Conclusion

The commercialisation of *iQhilika* has had an impact on employment creation in the Eastern Cape. The continued expansion and intensification of this process has the potential to contribute to the sustainable development of a functional rural economy in the Eastern Cape.

7.5 Concluding remarks

The research programme discussed in this thesis has succeeded in elucidating the underlying biotechnological principles within the Khoi-San/Xhosa *iQhilika* IKS, and allowing for the commercialisation of this traditional mead based on a firm scientific foundation. In addition to creating financial interest in the traditional beverage, this programme has demonstrated that environmental and social interests can also be served through the commercialisation of IKS. This commercialisation process has successfully occurred elsewhere in the past for other traditional beverages such as beer and wine.

The research programme culminated in the establishment of the Makana Meadery and, in this way validated the research hypothesis that the commercialisation of *iQhilika* may be based on both an understanding of the social functions of the beverage and its production, and the scientific elucidation of the underlying traditional biotechnological principles.

In completion of this objective *iQhilika* produced by Makana Meadery was chosen to be the welcoming drink at the inauguration of the President of South Africa, Mr Thabo Mbeki in 2004. It is believed that this is the first time that a commercially produced indigenous African beverage has been served at the inauguration of a president in this country, and represents a model for IKS recovery and the use of biotechnology in the development of rural economies.

7.6 Future work

Future work:

In many respects the data presented in this thesis represents the first scientific description of the traditional *iQhilika* process and its components and also the first description of a commercial Meadery established in South Africa to produce *iQhilika*. This may provide a model for future commercialisation of other mead varieties in Africa which would ensure the survival of this culturally significant sector of traditional African biotechnology.

The primary objective of the research effort was to understand some aspects of the biological activity underpinning the process, however, many questions remain to be answered and have had to be left for future research efforts and include a wide range of activities.

The origin of yeast in *iQhilika* is unknown. It is known that the same yeast is used to make *iQhilika* in two areas geographically distinct from each other, but it is unknown whether this yeast is derived from the roots of *Trichodiadema* sp. plants, from bees, from humans or from some other part of the environment. If the origin of the yeast could be determined, then a comprehensive survey could be undertaken to isolate

and evaluate additional yeast isolates against the current strains and ensure continued strain improvement.

Secondary maturation fermentations may be carried out by some of the LAB isolated. It would be interesting to test individual isolates in batches of *iQhilika* to see if, as in wine, LAB do contribute organoleptic complexity to the mead.

The use of bee brood and pollen in mead and its contribution to flavour are of interest. The ingredients represent a source of complex organic molecules, proteins, lipids, colour compounds and vitamins. It is likely that these can contribute to the overall organoleptic properties of *iQhilika* and further development of an understanding of how this occurs would be important to the ongoing development and improvement of the product.

The continued operation of the tower bioreactor systems will allow improvement of the control of the systems, possibly including computerised control. This would enable the flow rate to be controlled to produce product of greater consistency and may also allow for computer interventions in the oscillatory fluctuations of yeast cells. If it is possible to produce three or four different populations of yeast oscillating at different rates it may be possible to remove the oscillation in product leaving the bioreactor. This may improve organoleptic properties.

The tower bioreactors are also enrichment processors and over time their continued operation will select for more flocculent, alcohol tolerant, strains of yeast, hence constant monitoring of the evolution of yeast within the tower bioreactor will be valuable.

The processing of must before fermentation, and the processing of product after fermentation, are all areas where further research will be useful. The analysis of flavour in must, and how it is affected by fermentation, will provide interesting data to explain what characterises *iQhilika*. The maturation of *iQhilika*, and mead in general, is also a largely unknown area. It is not known by what mechanisms mead matures, but it is evident that with time mead improves. Wine improves with age to a point and then deteriorates. Mead does not spoil as fast as wine, when opened, and it is

possible that mead will improve for considerably longer than wine as a consequence. This requires further study.

Many apidological and apicultural questions may also be asked. Mead making requires an entirely different grade of honey to commercial honey packing. The extraction of unripe honey may produce mead with greater fermentability and stronger flavour. The careful analysis of mead made at a bench scale with different types of honey can allow for better operation of the commercial scale bioreactor with that honey. Different honey types will produce different types of mead, some of which may be unpleasant. Chemical characterisation of mead to assess its usefulness in mead making will avoid costly utilization of honey in mead making that would have been better sold or used for another product.

The conversion of mead to secondary products such as mead vinegar has been evaluated on pilot scale (not reported in this thesis). The development of secondary mead products such as sweetened mead vinegar, honey mustard with mead vinegar, mead pickles, mead vinegar and fruit chutney, mead vinegar salad dressings and various other products can all create an additional demand for mead. These products require further research and development of market acceptance.

Traditional *iQhilika* brewers make *iQhilika* with honey, or honey and additives such as fruits. The production of experimental mead with various indigenous and exotic fruit has been tested briefly, but needs to be researched further. Many indigenous fruits, as well as pineapples, contain malic acid. The potential of these fruits to stimulate malo-lactic fermentation in *iQhilika*, and thus contribute additional layers of sensory complexity to the beverage, is of interest.

The continuous *iQhilika* bioreactor system is also potentially applicable to fuel ethanol production. Sunflowers represent a good beekeeping crop which produces copious honey and the seeds are rich in oil. Sugar beet juice can be fermented to ethanol in the continuous *iQhilika* tower bioreactor. Bio-diesel can be produced from sunflower oil and ethanol. Rough estimates suggest that 60 hectares of sunflowers and 25 hectares of sugar beet could provide sufficient feedstock to fuel a beekeeping operation of 1000 hives. In addition the sunflowers are good for the

bees. This type of integrated management operation would allow a Meadery to reduce its overall impact on the environment.

The cultural aspects of *iQhilika* need to be recorded from various perspectives, scientific, sociological, economic, anthropological and even archaeologically to preserve the significance of this ancient beverage for future generations.

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