

https://dspace.org/

Enzymatic hydrolysis of bitter sorghum for bioethanol production

Deenanath, Evanie Devi

2010-08-24
http://hdl.handle.net/10539/8544

Downloaded from a DSpace repository. https://dspace.org/

ENZYMATIC HYDROLYSIS OF BITTER SORGHUM FOR BIOETHANOL PRODUCTION

Evanie Devi Deenanath

A Dissertation submitted to the Faculty of Engineering and the Built Environment, University of the Witwatersrand, Johannesburg, South Africa, in fulfillment of the requirements for the degree of Master of Science in Engineering.

Johannesburg 2009

DECLARATION

I declare that this research report is my own work and that sources that I have used or quoted have been indicated and acknowledged by means of complete references. It is submitted for the Degree of Masters of Science in Engineering in the University of Witwatersrand, Johannesburg.

_____

Candidate: E. D. Deenanath

_____ Month of _____ 2009

FACULTY OF ENGINEERING AND THE BUILT ENVIRONMENT.

UNIVERSITY OF THE WITWATERSRAND.

MASTERS OF SCIENCE IN ENGINEERING (MSc. Eng)

ENZYMATIC HYDROLYSIS OF BITTER SORGHUM FOR BIOETHANOL PRODUCTION

ABSTRACT

The production of bioethanol derived from biomass and fermentation is becoming increasingly popular due to the application in the motor-fuel industries. The present work, therefore aimed to investigate level of bioethanol produced from bitter sorghum grains by the use of commercial exogenous enzymes and to evaluate the microbial quality of sorghum processing. A temperature-programmed mashing regime was carried out using a dual-enzyme combination of Cerezyme Sorghum and Fungamyl 800L for the hydrolysis of sorghum starch. Subsequently, bioethanol was produced by fermenting the hydrolyzate with either *Saccahormyces cerevisiae* strain NRRL Y2084 or *Issatchenkia orientalis*. HPLC analyses of the hydrolyzates showed the presence of fructose, glucose and maltose. This indicated that bitter sorghum grains can be converted to fermentable sugars using these particular enzymes. Both yeast species are capable of fermenting the available sugars, producing 7% (v/v) of alcohol.

At the post-mashing stage, no microbial contaminants were found to be associated with the process. At the post-fermentation stage, plate counts showed microbial counts between 5.00-8.00 Log cfu/mL. The characterization of microbes isolated at the post-fermentation stage was based on PCR amplification of the 16S and ITS regions.

Following sequencing, the isolates were identified as *Lactococcus lactis, Lactcoccus garvieae, Lactobacillus casei, Enterococcus faecalis, Saccharomyces cerevisiae* strains NCL117 and T8, *Saccharomyces paradoxus, Saccharomyces pastorianus, Saccharomyces kudriavezvii, Issatchenkia orientalis* and *Candida inconscipua.* Fermentation conditions were favourable for the survival of these MOs.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude, indebtedness and appreciation to the following persons whose contribution, inspiration and support made this research possible:

- **The Almighty God,** for inner strength.
- **My parents, Mr & Mrs Deenanath,** for their unconditional love, support, motivation, and sacrifice throughout my scholastic and academic journey.
- **Professor Herman Potgieter,** for granting me permission to pursue my Masters Degree in the School of Chemical and Metallurgical Engineering, Wits University.
- **Professor Sunny Iyuke, my supervisor,** for the many hours spent offering advice, encouragement, suggestions and guidance. Your contributions have been invaluable and inspiring. Without your involvement and expertise in research, the completion of this project would not have been possible.
- **Dr. Denise Lindsay, my co-supervisor,** for trusting my abilities to undertake a research project in Engineering. It was a dream come true!
- **APV Invensys and NRF-Grant for Bioprocess Engineering in Nanotechnology Flagship,** for funding. The generous offers enabled me to conduct the research.
- **Falcon Engineering,** for design and supply of the Microbrewery Plant.

- **Mr. John Cluett (IBD-Africa Section), Mr. Anton Erasmus (Trade Brewer-SAB Alrode Plant) and Mr. Andrew Downes (Plant Manager of SAB Training Institute),** for technical support and assistance.
- **Mr. John Cluett (IBD-Africa Section),** for giving me the opportunity to further my knowledge in Brewing Science by allowing me to pursue a Diploma in Brewing through the Institute of Brewing and Distilling, UK (IBD).
- **ECOLAB,** for the donation of CIP-Cleaning and Disinfectant facility.
- **Mr. Donavin Hawker (Novozymes-SA),** for the kind donation of enzymes used to conduct the experiments.
- **TigerBrands,** for the supply of sorghum grains used for experimental purposes.
- **Mr. Christiaan Labuschagne (Inqaba Biotechnical Industries-SA),** for DNA extractions and PCR amplification of yeast isolates and sequencing of all samples.
- **Fellow postgraduate students,** for making my experience in Chemical Engineering unforgettable, interesting and loads of fun!

TABLE OF CONTENTS

DECLARATION **i**

ABSTRACT **ii**

ACKNOWLEDGEMENTS **iv**

TABLE OF CONTENTS **vi**

LIST OF FIGURES **x**

LIST OF TABLES **xiv**

LIST OF ABBREVIATIONS **xv**

CHAPTER 1: INTRODUCTION

(1.1) Background and Motivation **1**

(1.2) Hypothesis **3**

(1.3) Justification of the Study **3**

(1.4) Scope of the Project **4**

(1.5) Specific Aims and Objectives **5**

(1.6) Expected Contribution to the Knowledge **5**

(1.7) Dissertation Outline **6**

(1.8) References **8**

CHAPTER 2: BIOETHANOL PRODUCTION

(2.1) Literature Review **11**

2.1.1. Overview of Bioethanol **11**

2.1.1.1. Introduction to Bioethanol **11**

2.1.1.2. The Production of Bioethanol **12**

2.1.2. Sorghum and Bioethanol Production **16**

2.1.2.1. An Overview of Sorghum **16**

2.1.2.2. Sorghum and Beer Brewing **17**

2.1.2.3. The Brewing of Sorghum **18**

2.1.2.4. The Processing of Sorghum **20**

2.1.3. The Use of Sorghum in Ethanol Biotechnology **21**

2.1.3.1. The Production of Bioethanol from Sorghum **21**

(2.2) Methodology **24**

2.2.1. Experimental Design **24**

2.2.2. Experimental Approach **25**

2.2.2.1. Grain Preliminary Tests **25**

2.2.2.2. Isolation of a Wild Yeast Species **25**

2.2.2.3. Yeast Culturing and Viability **26**

2.2.2.4. Sorghum Processing for Bioethanol Production **27**

2.2.2.5. Analytical Methods **32**

(2.3) Results and Discussion **35**

2.3.1. Grain Preliminary Tests **35**

2.3.2. Identification of Yeast Isolates **35**

2.3.3. Yeast Pitching Rate **36**

2.3.4. Sorghum Processing for Bioethanol Production **37**

(2.4) Conclusions **47**

(2.5) References **48**

CHAPTER 3: MICROBIOLOGICAL ANALYSIS

(3.1) Literature Review **53**

3.1.1. Microorganisms Associated with Mashing and Fermentation Processes **53**

3.1.2. Gram Positive Spoilage Bacteria **53**

3.1.2.1. The Genus Lactobacillus **54**

3.1.2.2. The Genus Pediococcus **55**

3.1.2.3. The Genus Micrococcus **55**

3.1.3. Gram Negative Spoilage Bacteria **56**

3.1.3.1. The Genus Pectinatus **56**

3.1.3.2. The Genus Megasphaera **56**

3.1.3.3. Additional Gram Negative Bacteria **57**

3.1.4. Wild Yeasts in Beer Spoilage **58**

3.1.5. Spoilage Microorganisms Associated with Sorghum Processing **59**

3.1.6. Detection of Beer Spoilage Microorganisms **60**

3.1.6.1. Detection of Gram Positive Bacteria **60**

3.1.6.2. Detection of Gram Negative Bacteria **61**

3.1.6.3. Detection of Wild Yeasts **61**

(3.2) Methodology **64**

3.2.1. Experimental Design **64**

3.2.2. Experimental Approach **65**

3.2.2.1. Sample Collection for Microbial Analysis **65**

3.2.2.2. Sample Preparation for Microbial Analysis **65**

3.2.2.3. Enumeration and Isolation of Microbial Populations **66**

3.2.2.4. Identification Strategy of Microbial Isolates **67**

(3.3) Results and Discussion **75**

3.3.1. Microbial Counts **75**

3.3.2. Identification of Microbial Populations **77**

3.3.2.1. Preliminary Identification **77**

3.3.2.2. Molecular Identification **83**

3.3.2.3. Characterization of Microbial Isolates **85**

(3.4) Conclusions **92**

(3.5) References **93**

CHAPTER 4: CONCLUDING REMARKS and FUTURE RECOMMENDATIONS

(4.1) Concluding Remarks **97**

(4.2) Future Recommendations **98**

APPENDIX A: PUBLICATIONS, PRESENTATIONS and OTHER ACTIVITIES DURING THE INVESTIGATION **99**

APPENDIX B: RECIPES for MEDIA, STAINS and SOLUTIONS **101**

APPENDIX C: PCR CYLCES OF YEAST AMPLIFICATION **108**

APPENDIX D: RAW DATA **109**

LIST OF FIGURES

Figure 2.1	Sweet Sorghum Grains of *Sorghum bicolor* **{L.}** Moench variety.
Figure 2.2	Schematic Diagram representing an outline of the Experimental Design.
Figure 2.3	The Wits Microbrewery Plant-designed and supplied by Falcon Engineering (Pty) Ltd, South Africa.
Figures 2.4 a-b	Red Swazi Variety of Sorghum Grains. (a) Un-malted sorghum grains. (b) Milled sorghum grains.
Figures 2.5 a-d	Brewhouse and Cold Liquid Tank of the Wits Microbrewery Plant.
Figure 2.6	Conical Fermentation Tank (100L) of the Wits Microbrewery Plant.
Figure 2.7	HPLC Chromatogram of five carbohydrate standards. Peak 1: Fructose, Peak 2: Glucose, Peak 3: Sucrose, Peak 4: Maltose, Peak 5: Lactose.
Figure 2.8	The Alcohol Gay-Lussac % (v/v) of standard mixtures of alcohol ranging from 5-20% (v/v).
Figure 2.9 a	Colonies of wild yeast isolate on PDA.
Figure 2.9 b	Grams stain of wild yeast isolate.
Figure 2.10	HPLC Chromatogram of the post-mashing sample with the addition of exogenous enzymes of sorghum processing one (SP1). Peak identification and retention times are indicated on the chromatogram according to the order of elution from the Zorbax Carbohydrate Column. Peak 1: Fructose, Peak 2: Unknown, Peak 3: Glucose, Peak

4: Maltose.

Figure 2.11 HPLC Chromatogram of the post-mashing sample without the addition of exogenous enzymes (Control). Peak identification and retention times are indicated on the chromatogram according to the order of elution from the Zorbax Carbohydrate Column. Peak 1: Fructose, Peak 2: Unknown, Peak 3: Unknown, Peak 4: Glucose, Peak 5: Maltose.

Figure 2.12 Maximum ethanol production by *S. cerevisiae* and *I. orientalis* of hydrolyzed starch obtained from bitter sorghum grains and exogenous enzymes. SP1: indicates fermentation by *S. cerevisiae* of enzymatically hydrolyzed starch. SP 2: indicates fermentation by *I. orientalis* of enzymatically hydrolyzed starch. Control: fermentation by S. cerevisiae of hydrolyzate obtained without the addition of exogenous enzymes.

Figure 3.1 Schematic Diagram representing an outline of the Experimental Design.

Figure 3.2 PCR Cycles of 16S rDNA Amplification

Figure 3.3 Log CFU/mL of bacterial and yeast cells on various types of media at the post-fermentation stage. Error bars indicate standard deviation between triplicate plate counts ($p<0.05$).

Figures 3.4 a-e Various types of media showing microbial populations associated with sorghum processing at the post-fermentation stage of SP 1. (a) UBA, (b) DM, (c) MRS, (d) PDA, (e) WLN. (a-c) bacterial colonies

as UBA, DM and MRS are media specific for the visualization of bacterial growth. (d-e) yeast and wild yeast colonies as PDA and WLN are media specific for the visualization of yeast growth.

Figures 3.5 a-e Various types of media showing microbial populations associated with sorghum processing at the post-fermentation stage of SP 2. (a) UBA, (b) DM, (c) MRS, (d) PDA, (e) WLN. (b-c) bacterial colonies as DM and MRS are media specific for the visualization of bacterial growth. (a, d, e) yeast and wild yeast colonies as UBA, PDA and WLN are media specific for the visualization of yeast growth.

Figures 3.6 a-e Gram Stains of isolates. (a) gram positive cocci, (b) gram positive rods, (c) budding yeasts, (d) elongated rods, (e) spherical yeasts.

Figure 3.7 Agarose Gel of 1500 bp DNA fragments amplified by 16S rDNA primers of UBA (SP 1), DM and MRS isolates. Lane 1: Negative Control. Lane 2: Positive Control. Lane 3: MRS isolate. Lane 4: Molecular Weight Marker (1kb DNA Ladder-Fermentas).

Figure 3.8 Phylogeny of 16S region. The tree is represented as a rooted tree with branch lengths computed by using DNAMAN. The scale bar is a statistical value as a result of a bootstrap value of 100. Isolates from this study is indicated in BLUE. BLAST identified sequences are indicated in GREEN. Sequences obtained from GenBank are indicated in PURPLE.

Figure 3.9 Phylogeny of 5.8S region. The tree is represented as a rooted tree with branch lengths computed by using DNAMAN. The scale bar is a

statistical value as a result of a bootstrap value of 100. Labeling is an indicated in Figure 3.8.

LIST OF TABLES

Table 2.1	The ten leading countries involved in bioethanol production, bioethanol content is expressed as billion gallons.
Table 2.2	A list of various feedstocks used for bioethanol production and the amount of bioethanol that can be produced from each type of feedstock.
Table 2.3	Summary of commercial enzymes from Novozymes.
Table 2.4	Quantitative Analysis of Carbohydrates as detected by HPLC.
Table 3.1	Incubation conditions of the various growth media used for sample analysis
Table 3.2	Incubation conditions of the liquid broth culture used for the isolation of pure colonies from each type of media.
Table 3.3	A list of bacterial species and GenBank accession numbers.
Table 3.4	A list of yeast species and GenBank accession numbers.
Table 3.5	Preliminary Characteristics of SP 1 isolates based on colony morphology, grams reaction, cell shape and catalase reaction.
Table 3.6	Preliminary Characteristics of SP 2 isolates based on colony morphology, grams reaction, cell shape and catalase reaction.
Table 3.7	Sequence identification of bacterial and yeast isolates from different types of media of SP 1 and SP 2.

LIST OF ABBREVIATIONS

ICEs: Internal Combustion Engines

MOs: Microorganisms

MO: Microorganism

dH_2O: Distilled Water

UBA: Universal Beer Agar

DM: Differential Medium

MRS: de Man, Rogosa & Sharpe Agar

PDA: Potato Dextrose Agar

WLN: Wallenstein Laboratory Nutrient Medium

BPW: Buffered Peptone Water

TSB: Tryptone Soy Broth

YPD: Yeast Peptone Dextrose

TBE: Tris borate EDTA buffer

HPLC: High Performance Liquid Chromatography

PCR: Polymerase Chain Reaction

BLAST: Basic Local Alignment Search Tool

LAB: Lactic Acid Bacteria

FAN: Free Amino Nitrogen

ITS: Internal Spacer Region

CHAPTER 1:
INTRODUCTION

(1.1) Background and Motivation

Bioethanol is a type of biofuel derived from biomass feedstocks such as sorghum, sugar cane, barley, corn, wheat, wood and straw (Balat *et al*., 2008). The production of bioethanol through bioprocessing, namely, acid or enzymatic hydrolysis and subsequent fermentation is becoming increasingly popular due to the application in Bio-Industries. Chemical and motor-fuel industries can utilize bioethanol (Suresh *et al*., 1999) as a fossil fuel substitute (Amigun *et al*., 2008). The use of bioethanol has several advantages such as:- reduction of combustion emissions, it is biodegradable, and accessibility from renewable resources (Balat *et al*., 2008; Demirbas, 2008).

Previously, maize or corn meal has proven successful for bioethanol production. Maize has a 64-78% starch content and is involved in approximately 98% of bioethanol production in the USA (Taylor *et al*., 2006). Over recent years attention has shifted towards sorghum grains as an alternative starch-based material for bioethanol production. The potential of sorghum for production of bioethanol is due to: (i) sorghum grains have a starch composition of 60-77%, (ii) it is a cheap starting material, and (iii) easily available as it is cultivated in large quantities (Odibo *et al*., 2002; Taylor *et al*., 2006). The use of sweet sorghum varieties have been exploited in the production of bioethanol. However, there is growing interest in utilization of bitter sorghum (bird-proof varieties) for bioethanol production (du Preez *et al*., 1985; Suresh *et al*., 1999; Omidiji and Okpuzor, 2002; Taylor *et al*., 2006). Bitter sorghum is usually high in tannins. This is

beneficial for agronomic purposes as tannin levels provide resistance to seed germination and molding prior to harvest (Waichungo and Holt, 1995). In addition, bitter sorghum has a high starch content as well as high alpha (α)- and beta (β)- amylase activity and hence is desired for bioethanol production (Dicko *et al*., 2006).

The escalating interest in bioethanol as an alternative to petroleum fuel, as the abundance of crude oil decreases, motivated this investigation. In recent times there has been an extensive decline in resources of fossil fuels, even though increased demand has resulted in increased carbon emissions leading to a polluted environment, and potentially contributing to global warming. If bioethanol can be produced and commercialized to the maximum extent, then it can replace fossil fuels, and in turn reduce environmental pollution as well as contribute to the decline in crude oil availability. In addition, the use of sorghum grains as a starting material is not a limiting factor in the production of bioethanol, as sorghum is extensively grown in numerous African countries. Sorghum is mainly used in the production of traditional African sorghum beer (Pattison *et al*., 1998) and thus is readily available and can be exploited in other industrial processes.

Furthermore, biological stability is a requirement in production processes for bioethanol, as contamination of hydrolysis and fermentation with contaminant microorganisms contributes to a decrease in bioethanol productivity (Basilio *et al*., 2008). For the purpose of this investigation, the detection of microbial contaminants is necessary to conclude which microorganisms are associated with bitter sorghum processing for bioethanol

production and if the contaminants are in fact those which negatively affect the bioethanol yield.

(1.2) Hypothesis

The use of bitter sorghum grains, in the un-malted form, with the addition of commercial exogenous enzymes processed in a microbrewery plant is efficient the production of fermentable sugars.

(1.3) Justification of the Study

Various studies to date have reported on the use of bitter sorghum grains for bioethanol production (du Preez *et al*., 1985; Mamma *et al*., 1995 & 1996; Suresh *et al*., 1999; Kim & Dale, 2004; Mojovic *et al*., 2006; Linde *et al*., 2008) However, the production of bioethanol utilizing a basic microbrewery plant has not been reported.

Biofuels, in general is a worldwide phenomenon that is considered to be a replacement source of fuel for internal combustion engines. In Africa, there are several problems with regards to fuel as the African continent is dependent on imports for their supply (Amigun *et al*., 2008). There are numerous resources that can be used to combat this problem. For example, the industrial utilization of sorghum is an option, as it is readily cultivated in Africa. If fermentation of sorghum grains in a microbrewery plant is capable of producing sugar substrates efficient for ethanol fermentation and a satisfactory yield of bioethanol can be achieved, then the implementation of large scale production will be of interest to Bio-Industries. Bio-Industries essentially offer environmental and economic

benefits by applying microorganisms (MOs) to convert renewable resources into bioenergy (Hall and Crowther, 1998; Prasad *et al*., 2007).

Furthermore, microbial contaminants are an important aspect of the microbrewery plant housed at the University of the Witwatersrand as a processing plant, as it is an indication of productivity, hygienic operational processes and maintenance of a full-scale plant. This research is therefore expected to provide information regarding the use of a microbrewery plant as a reliable means of bioethanol production. This will bring about future economical benefits by possibly becoming a major contributing factor to the net productivity of bioethanol.

(1.4) Scope of the Project

The ultimate goals of this research are to:

(a) Investigate the production of bioethanol using un-malted bitter sorghum grains and exogenous enzymes

(b) Evaluate microbiological contamination throughout the process.

Therefore, the scope of this research is based on the following questions:-

(1) Is the addition of the external commercial enzymes, i.e. Cerezyme Sorghum and Fungamyl 800L sufficient to produce fermentable sugars from un-malted sorghum grains using a microbrewery plant?

(2) Would the enzymes increase the concentration of fermentable sugars via hydrolysis compared to non-enzymatic hydrolysis?

(3) Which microorganisms are inhabitants of the microbrewery plant housed at the University of the Witwatersrand during sorghum processing?

(4) Are the isolated microbes related to previously detected microbes associated with hydrolysis and fermentation processes using sorghum as a raw material for bioethanol production?

(1.5) Specific Aims and Objectives

The specific aims of this study is to investigate the level of bioethanol produced from bitter sorghum grains, using a combination of commercial exogenous enzymes and two types of yeast species to obtain maximum ethanol by fermentation. In addition, the microbiological quality at various stages of sorghum processing will be examined, using the microbrewery plant housed at the University of the Witwatersrand.

The aims will be achieved by the following objectives:-

(i) To analyze the fermentable sugars obtained from un-malted bitter sorghum grains by a dual-enzymatic hydrolysis.

(ii) To detect the level of ethanol produced from the hydrolyzate during fermentation by *S. cerevisiae* and *I. orientalis.*

(iii) To evaluate the presence of microbiological contamination during sorghum processing and fermentation of enzymatic hydrolyzates.

(iv) To characterize microbial isolates obtained from sorghum processing.

(1.6) Expected Contribution to Knowledge

This report will contribute knowledge towards:- (1) Biotechnology-related industries in Africa and worldwide with regards to the use of bitter sorghum grains as a way of obtaining bioethanol from a microbrewery plant housed at the University of the Witwatersrand and (2) Biological stability in relation to improved bioethanol productivity and controlling contamination through hygienic maintenance of a brewery plant.

(1.7) Dissertation Outline

CHAPTER 1:

Chapter 1 is a general introduction sub-divided into:- Background and Motivation, Hypothesis, Justification of the Study, Scope of the Project, Specific Aims and Objectives, and Expected Contribution to Knowledge.

CHAPTER 2:

Chapter 2 is entitled: Bioethanol Production. This chapter is divided into:- Literature Review, Methodology, Results and Discussion, Conclusions and References. The Literature Review provides background knowledge on bioethanol and sorghum. Methodology is sub-divided into Experimental Design and Experimental Approach. Experimental Design is a flow diagram briefly outlining the bioethanol production process, followed by in-depth details of the experimental procedures under the heading, Experimental Approach. In addition, the isolation of *I. orientalis* and grain preliminary tests are included in the Methodology Section. Results are represented as Figures and Tables, which are then interpreted and discussed with comparisons to previous research

studies. Conclusions are described in point form to summarize the findings of the research. References are presented alphabetically. All references that appear in this section are cited in the text.

CHAPTER 3:

Chapter 3 is entitled: Microbiological Analysis. This chapter is divided into:- Literature Review, Methodology, Results and Discussion and Conclusions and References. The Literature Review provides background knowledge on microorganisms in relation to the beer industry and microorganisms associated with sorghum beer brewing. Methodology is sub-divided into Experimental Design and Experimental Approach. Experimental Design is an outline of standard microbiological procedures and molecular methods. Experimental Approach provides details of the experimental procedures used. Results are represented in Figures and Tables, which are then interpreted and discussed with comparisons to previous research studies. Conclusions are described in point form to summarize the findings of the research. References are presented alphabetically. All references that appear in this section are cited in the text.

CHAPTER 4:

Chapter 4 is entitled: Concluding Remarks and Future Recommendations. This chapter provides an overall conclusion to the investigation and recommendations that contribute to these particular findings.

(1.8) References

Amigun, B., Sigamoney, R. and von Blottnitz, H. (2008). Commercialization of Biofuel Industry in Africa: A review. *Renewable and Sustainable Energy Reviews*. **12:** 690-711.

Balat, M., Balat, H. and Cahide, O. (2008). Progress in Bioethanol Processing. *Progress in Energy and Combustion Science.* **34:** 551-573.

Basilio, A.C.M., de Araujo, P.R.L., de Morais, J.O.F., da Silvio Filho, E.A., de Morais, M.A. and Simoes, D.A. (2008). Detection and Identification of Wild Yeast Contaminants of the Industrial Fuel Ethanol Fermentation Process. *Current Microbiology*. **56:** 322-326.

Demirbas, A. (2008). Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. Energy *Conversion and Management.* **49:** 2106-2116.

Dicko, M.H., Gruppen, H., Traore, A.S., Voragen, A.G.J. and Berkel, W.J.H. (2006). Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *African Journal of Biotechnology.* **5(5):** 384-395.

du Preez, J.C., de Jong, F., Botes, P.J. and Lategan, P.M. (1985). Fermentation Alcohol from Grain Sorghum Starch. *Biomass.* **8:** 101-117.

Hall, J. and Crowther, S. (1998). Biotechnology: the ultimate cleaner production technology for agriculture? *Journal of Cleaner Production.* **6:** 313-322.

Kim, S. and Dale, B.E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy.* **26:** 361-375.

Linde, M., Galbe, M. and Zacchi, G. (2008). Bioethanol production from non-starch residues in process streams from a dry-mill ethanol plant. *Bioresource Technology*. **99:** 6505-6511.

Mamma, D., Christakopoulos, P., Koullas, D., Kekos, D., Macris, B.J. and Koukios, E. (1995). An alternative approach to the bioconversion of sweet sorghum carbohydrates to ethanol. *Biomass and Bioenergy*. **8(2):** 99-103.

Mamma, D., Koullas, D., Fountoukidis, G., Kekos, D., Macris, B.J. and Koukious, E. (1996). Bioethanol from sweet sorghum: simultaneous saccharification and fermentation of carbohydrates by a mixed microbial culture. *Process Biochemistry*. **4:** 377-381.

Mojovic, L., Nikolic, S., Rakin, M. and Vukasinovic, M. (2006). Production of bioethanol from corn meal hydrolyzates. *Fuel*. **85:** 1750-1755.

Odibo, F.J.C., Nwankwo, L.N. and Agu, R.C. (2002). Production of malt extract and beer from Nigerian Sorghum Varieties. *Process Biochemistry*. **37:** 851-855.

Pattison, T., Geornaras, I., and von Holy, A. (1998). Microbial populations associated with commercially produced South African sorghum beer as determined by conventional and petrifilm plating. *International Journal of Food Microbiology*. **43:** 115-122.

Prasad, S., Singh, A. and Joshi, H.C. (2007). Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Resources, Conservation, and Recycling*. **50:** 1-39.

Suresh, K., Kiransee, N. and Rao, L.V. (1999). Production of ethanol by raw starch hydrolysis and fermentation of damaged grains of wheat and sorghum. *Bioprocess Engineering*. **21:** 165-168.

Taylor, J.R.N., Schober, T.J. and Bean, S.R. (2006). Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science.* **44:** 252-271.

Waichungo, W.W. and Holt, D.L. (1995). Use of Ammonium Hydroxide to Reduce the level of Assayable Tannin in High-Tannin Sorghum Grain. *Journal of Agricultural Food Chemistry*. **43:** 728-732.

CHAPTER 2:
BIOETHANOL PRODUCTION

(2.1) Literature Review

2.1.1. Overview of Bioethanol

2.1.1.1. Introduction to Bioethanol

The term bioethanol can be defined as a biofuel produced from biomass via biochemical processes such as hydrolysis and fermentation (Demirbas, 2008). Biologically derived bioethanol is an ethyl alcohol or ethanol consisting of approximately 5% water and 35% oxygen (Balat *et al*., 2008; Demirbas, 2008). This type of alcohol is suitable for the motor-fuel industry as fuel for internal combustion engines (ICEs) (Demirbas, 2008) and is considered to be a "petrol additive" (Demirbas, 2008).

Bioethanol is a renewable oxygenated fuel source with a high octane number, high flame speeds, high heat of vaporization and a wide range of flammability limits (Balat *et al*., 2008; Sanchez and Cardona, 2008). These properties offer numerous advantages such as:- (i) improved oxidation of hydrocarbons, (ii) a decrease in carbon monoxide and hydrocarbon emissions and (iii) in an ICE, the compression ratio is higher and the burn time is shorter (Balat *et al*., 2008; Sanchez and Cardona, 2008). Hence, bioethanol as a fuel substitute is becoming increasingly popular in order to reduce environmental pollution and import of crude oil (Balat *et al*., 2008; Sanchez and Cardona, 2008).

Currently, the United States of America (USA) and Brazil contribute approximately 62-70% of the world's bioethanol production (Table 2.1) (Kim and Dale, 2004; Linde *et al*.,

2008). During 2006, in the USA, 4.30-4.85 billion gallons of bioethanol was produced (Balat *et al*., 2008; Linde *et al*., 2008). In Brazil the common feedstock used for bioethanol production is sugar cane and 24% of the ethanol produced is mixed with 76% of gasoline (Balat *et al*., 2008; Linde *et al*., 2008). Whereas in the USA, corn grain is commonly used for bioethanol production and 10% of the ethanol produced is mixed with 90% of gasoline (Balat *et al*., 2008; Linde *et al*., 2008).

Table 2.1. The ten leading countries involved in bioethanol production, bioethanol content is expressed as billion gallons (*Source*: Balat *et al*., 2008).

Country	2004	2005	2006
USA	3.54	4.26	4.85
Brazil	3.99	4.23	4.49
China	0.96	1.00	1.02
India	0.46	0.45	0.50
France	0.22	0.24	0.25
Germany	0.07	0.11	0.20
Russia	0.20	0.20	0.17
Canada	0.06	0.06	0.15
South Africa	0.11	0.10	0.10
Thailand	0.07	0.08	0.09

2.1.1.2. The Production of Bioethanol

Bioethanol production involves the hydrolysis of biomass feedstocks-which is the starting raw material, followed by fermentation using microorganisms (MOs) (Balat *et al* 2008; Sanchez and Cardona, 2008). There are numerous feedstocks available from which bioethanol can be derived (Table 2.2). These include:- (i) sucrose feedstocks, (ii) starch feedstocks and (iii) lignocellulosic feedstocks (Balat *et al*., 2008). The processing or hydrolysis into fermentable sugars varies depending on the type of feedstock used (Sanchez and Cardona, 2008). For fermentation, *Saccharomyces (S.) cerevisiae* is the

preferred microorganism (MO), due to its ability to easily convert sugars and its potential to proliferate anaerobically (Sanchez and Cardona, 2008).

Table 2.2. A list of the various feedstocks used for bioethanol production and the amount of bioethanol that can be produced from each type of feedstock (*Source:* Balat *et al*., 2008).

	Bioethanol production potential (l/ton)
Sugar cane	70
Sugar beet	110
Sweet potato	125
Potato	110
Cassava	180
Maize	360
Rice	430
Barley	250
Wheat	340
Sweet sorghum	60
Bagasse and other cellulose biomass	280

(i) Processing of Sucrose Feedstocks

Sugar beet, sweet sorghum and sugar cane are classified as sucrose feedstocks (Balat *et al*., 2008). Conversion of sucrose feedstocks involve the use of commercial enzymes such as amylases, cellulases and amylopectinases, resulting in yeast assimilable monosaccharides such as glucose and fructose (Sanchez and Cardona, 2008). Fermentation of sucrose feedstocks is by *S. cerevisiae.* In addition, the wild yeast species, *Schizosaccharoymces pombe* and the bacterial species, *Zymomonas mobilis* has been shown to be useful in the production of ethanol from sucrose. Batch or continuous fermentation processes can be carried out (Sanchez and Cardona, 2008).

(ii) Processing of Starch Feedstocks

Wheat, corn, barley and cassava are classified as starch feedstocks (Balat *et al*., 2008). Starch is a long chain polymer that requires enzymatic hydrolysis for the break down into single glucose units that can be utilized by yeasts (Balat *et al*., 2008). Starch hydrolysis involves liquefaction and saccharification. During liquefaction, the starch polymer is broken down into smaller glucose units and dextrines by amylase enzymes. This step is carried out at high temperatures ranging between 90-110°C (Sanchez and Cardona, 2008). Saccharification is the conversion of the liquefied starch via the action of the enzyme glucoamylase, at temperatures ranging between 60-70°C. Fermentation of starch feedstocks is carried out using *S. cerevisiae* (Sanchez and Cardona, 2008).

(iii) Processing of Lignocellulosic Feedstocks

Wheat straw, corn stover, wood and grasses are classified as lignocellulosic feedstocks (Balat *et al*., 2008). Lignocellulosics are complex feedstocks composed of three polymers, namely:- cellulose, hemicellulose and lignin (Balat *et al*., 2008). Processing of lignocellulosic feedstocks involves (a) pre-treatment, then (b) hydrolysis and (c) fermentation (Balat *et al*., 2008; Sanchez and Cardona, 2008).

(a) Pre-treatment

Pre-treatment is required to change the complex structure by removing the lignin and hemicellulose and reducing cellulose. This is necessary to produce accessible enzyme substrate for hydrolysis. Lignocellulosics can be pre-treated via chemical or biological methods (Balat *et al*., 2008; Sanchez and Cardona, 2008). Chemical pre-treatment utilizes dilute or concentrated acids such as sulphuric acid or hydrochloric acid. Dilute sulphuric acid pre-treatment involves the depolymerization of hemicellulose for 15 minutes at

140°C, followed by reduction of cellulose for 10 minutes at 190°C (Balat *et al*., 2008; Sanchez and Cardona, 2008). Biological pre-treatment involves the application of MOs, such as white-rot fungi which degrades the lignin (Sanchez and Cardona, 2008).

(b) Hydroylsis

Hydrolysis is the degradation of cellulose into glucose via acidic hydrolysis or enzymatic hydrolysis (Balat *et al*., 2008; Sanchez and Cardona, 2008). Acid hydrolysis involves the use of dilute or concentrated sulphuric acid or hydrochloric acid. Dilute acid hydrolysis is a two-stage process. The first stage is performed at 190°C for 3 minutes using a 0.7% acid concentration to produce pentoses. The second stage is performed at 215°C for 3 minutes using a 0.4% acid concentration to produce hexoses (Sanchez and Cardona, 2008). Concentrated acid hydrolysis uses 30-70% sulphuric acid for 10-12 hours (Sanchez and Cardona, 2008). Enzymatic hydrolysis utilizes various commercial cellulase enzymes obtained from *Trichoderma reesei* species. These cellulases are cellobiohydrolases, endoglucanases, β-glucosidases and hemicellulases (Sanchez and Cardona, 2008). Cellobiohydrolases break down crystalline cellulose, whilst endoglucanases break down amorphous cellulose. β-glucosidases hydrolyze cellobiose into glucose molecules (Sanchez and Cardona, 2008).

(c) Fermentation

S.cerevisiae is the preferred yeast as it is able to assimilate hexoses produced as well as inhibitory compounds formed from lignocellulosic hydrolyzates (Balat *et al*., 2008). However, the pentoses can not be assimilated. Thus, in addition, bacterial species such as *Z. mobilis, Escherichia coli* and *Klebsiella oxytoca* can be used to obtain efficient yields

of bioethanol from lignocellulosics. Fermentation parameters are optimized depending on the choice of MO (Balat *et al*., 2008).

2.1.2. Sorghum and Bioethanol Production

2.1.2.1. An Overview of Sorghum

Sorghum is a cereal plant which is a member of the family *Graminae* and grouped within the genus *Sorghum* (Palmer, 1992; Owuama, 1997). *Sorghum vulgara* and *Sorghum bicolor* are examples of species belonging to the genus *Sorghum* (Palmer, 1992). *Sorghum vulgara* are "annual sorghums" (Owuama, 1997) while *Sorghum bicolor* (Fig 2.1) are "cultivated sorghums" (Dicko et al., 2006).



Fig 2.1. Sweet Sorghum grains of the *Sorghum bicolor* {L.} Moench variety (*Source:* Dicko *et al*., 2006).

This angiosperm, tropical cereal plant is indigenous to Africa and extensively grown in Sub-Saharan Africa, with approximately 20 million tones of sorghum being produced annually (Taylor *et al*., 2003; Dicko *et al*., 2006). Semi-arid and tropical conditions in these regions are favourable for the growth of sorghum (Palmer, 1992; Dicko *et al*.,

2006) and sorghum plants are well-adapted to survive harsh drought conditions (Agu and Palmer, 1998). In addition, sorghum is grown in Asia, USA, South America, Australia, China and India (Dicko *et al*., 2006). In Africa, Asia and India sorghum is an important food source. (Palmer, 1992; Owuama, 1997; Agu and Palmer, 1998). Furthermore, sorghum is utilized for various other purposes such as:- animal feed and the production of industrial products-for example, alcohol and oil (Owuama, 1997; Dicko *et al*., 2006). Annual worldwide production of sorghum is approximately 60-65 million tones, making this particular plant the fifth most popular cereal plant after wheat, rice, maize and barley (Palmer, 1992; Dicko *et al*., 2006).

Sorghum is a suitable food cereal as it is rich in vitamins, minerals, carbohydrates, proteins and fats (Dicko *et al*., 2006). In developing countries, approximately 300 million people depend on sorghum as a food supplement (Dicko *et al*., 2006). Examples of foods produced using sorghum are:- tortilla, thin porridge, couscous, injera, nasha and traditional beers (Dicko *et al*., 2006).

2.1.2.2. Sorghum and Beer Brewing

The potential of sorghum in the brewing of beer is of great interest for the following reasons:- (i) sorghum is an abundant source of starch and proteins, (ii) cheap starting material and (iii) easily available as it is cultivated in large quantities (Odibo *et al*., 2002). In Africa, traditional beer brewed using sorghum is called opaque beer (Agu and Palmer, 1998; Taylor *et al*., 2003). Opaque beer is pinkish-brown in colour, sour in taste and viscous (Pattison *et al*., 1998). This beer consists of an alcohol content of ~3%, with

variable amounts of insoluble starch and dextrines (Agu and Palmer, 1998; Pattison *et al*., 1998). Furthermore, opaque beer is unpasteurized and usually consumed in an "active state of fermentation" (Taylor *et al*., 2003). Currently, 13 million hectoliters of opaque beer is brewed annually in various parts of Southern and Central Africa (Taylor *et al*., 2003). Zimbabwean opaque beer is referred to as *doro, hwahwa, mhamba* or utshwala (Bvochora and Zvauya, 2001). Traditional Zimbabwean beer brewing is unique, utilizing sorghum grains that are high in tannins or polyphenols. The end-product is usually liquid or semi-solid with an alcohol content of between 1-4% (Bvochora and Zvauya, 2001). The alcohol is produced by "spontaneous lactic acid and alcoholic fermentation stages" (Bvochora and Zvauya, 2001). The use of sorghum is also extensively exploited in the production of lager and stout beers in Nigeria and South Africa, not only in traditional opaque beer (Dicko *et al*., 2006; Taylor *et al*., 2006), and has escalated into large scale commercial production (Taylor *et al*., 2006).

2.1.2.3. The Brewing of Sorghum

Sorghum beer production involves malting, souring, boiling, mashing, straining and alcoholic fermentation (Pattison *et al*., 1998).

Malting of different sorghum varieties results in different proportions of α- and β-amylases (Owuawa, 1997). For the malting step, sorghum grains are soaked in water for 24h at 25°C. The grains are then allowed to germinate for 6-8 days at 25°C. During germination, the grains are continuously hydrated by spraying with water. Following germination the grains are kilned for 24h at temperature ranging between 45-65°C (Owuawa, 1997). Kilning causes a reduction in the enzyme content of the malt (Palmer,

1992; Owuawa, 1997). In addition, malt sugar content is affected (Owuawa, 1997). To minimize the enzyme reduction, the malt is kilned via two stages. During the first stage, the malt is dried at 55°C. At the second stage, the temperature is increased to 65°C (Owuawa, 1997). The increase in temperature results in optimal recovery of enzymes and higher sugar concentrations (Owuawa, 1997). Maximum amount of sorghum malt is extracted by removing the sorghum malt and recombining the extract enzymatic malt with gelatinized starch at 65°C (Palmer, 1992).

Souring or lactic acid fermentation is due to the metabolic activity of *Lactobacillus,* producing a sour taste. Post-lactic acid fermentation, continuous lactic acid production is prevented by boiling (Pattison *et al*., 1998).

Mashing of sorghum malt by the three-stage decoction process is efficient. Initially, 70% of the malt is mashed. During the second stage, mashing occurs for 30min at 65°C. At the third stage, mashing occurs for 30-60min at 70°C (Owuawa, 1997). This process allows for maximum wort hydrolysis (Owuawa, 1997). Furthermore, during mashing, an external enzyme can be added to increase the yield of wort extracted (Owuawa, 1997).

Alcoholic fermentation involves pitching the wort with dry yeast. *S.cerevisiae* is typically used (Pattison *et al*., 1998).

2.1.2.4. The Processing of Sorghum

In comparison to barley, sorghum requires additional processing for its use in brewing. Optimal conditions used for barley are not necessarily optimal for sorghum. Starch gelatinization and an increase in β-amylase activity of sorghum are two aspects that are important to obtain maximum yields of fermentable sugars (Taylor *et al*., 2006).

(a) Gelatinization of starch

Initially, sorghum grains or malt are cooked at temperatures ranging between 67-73°C for the gelatinization of starch (Taylor *et al*., 2006). The gelatinized starch is then hydrolyzed by barley malt or external enzymes (Taylor *et al*., 2006). Gelatinization temperatures differ amongst sorghum varieties. For example- Barnard Red, a South African sorghum variety gelatinizes starch at 59.4°C. Waxy sorghum, on the other hand, gelatinizes quicker at a temperature of 69.6°C and is easily hydrolyzed by amylases (Taylor *et al*., 2006).

(b) Increasing Beta-amylase activity

The hydrolytic enzyme β-amylase is synthesized when sorghum grains are germinated. However, the activity of sorghum β-amylase is approximately 25% less than barley (Taylor *et al*., 2006). To improve the activity of this enzyme, malting conditions are varied. During the steeping step of malting, air-rests and an additional step of steeping the grains in 40°C water results in increased β-amylase activity. Furthermore, the diastatic power of sorghum, which is a combination of α- and β-amylase activity, is increased when the grains are steeped in 0.1% NaOH or 0.1% $Ca(OH)_2$ (Taylor *et al*., 2006). For germination, high moisture and low temperature increases the activity of β-amylase (Taylor *et al*., 2006).

2.1.3. The use of Sorghum in Ethanol Biotechnology.

Sorghum grains have a starch composition of 60-77%. This abundance of starch is essential to the ethanol technolgy, as sorghum is an important aspect for the production of bioethanol (Taylor *et al.*, 2006). For example:- (1) cultivated sorghum contributes to 10-20% of bioethanol in the USA, with approximately 0.49-0.95 billion liters of bioethanol produced (Taylor *et al*., 2006) and (2) the distillery industries in India has switched from sugar cane molasses to sorghum for bioethanol production (Aggarwal *et al*., 2001).

2.1.3.1. The Production of Bioethanol from Sorghum

Previously, maize and corn meal has proved to be successful for bioethanol production. Maize has 64-78% starch content and is involved in approximately 98% of bioethanol production in the USA (Taylor *et al*., 2006). Bioethanol production of corn meal is via hydrolysis of the starch using α-amylase and glucoamylase. The hydrolyzates obtained are then fermented by *S.cerevisiae* to produce ethanol (Mojovic *et al*., 2006). The use of corn meal results in 80% (w/w) yield of ethanol (Mojovic *et al*., 2006).

Over recent years, the attention has shifted towards sorghum as an alternative starch-based material for bioethanol production. There are different varieties of sorghum grains that can be used. These are high-tannin or low tannin varieties (Waniska *et al*., 1992). Variations between the varieties affect the amount of ethanol production. All sorghum varieties are mainly composed of starch (Dicko *et al*., 2006). However, some sorghum varieties contain non-starch polysaccharides and phenolic compounds (Dicko *et al*., 2006; Dlamini *et al*., 2007). These compounds are associated with processing problems (Dicko *et al*., 2006; Dlamini *et al*., 2007).

For the purpose of bioethanol production, conversion of starch to fermentable sugars is required. Starch conversion is achieved by enzymatic hydrolysis. The enzymes α- and β-amylases are necessary for hydrolysis and are present within the sorghum grain endosperm (Palmer, 1992). Starch is composed of amylose and amylopectin, which are high molecular weight molecules. Amylose is a linear molecule made up of α-(1-4)-D-glucopyranose (Dicko *et al*., 2006). Amylopectin is a branched molecule made up of α-(1-4)-D-glucopyranose and α-(1-6)-D-glucopyranose (Dicko *et al*., 2006). During starch hydrolysis α- and β-amylases act on the α-(1-4) linkages. Alpha-amylases are endoenzymes and are involved in starch liquefaction. Beta-amylases are exoenzymes and are involved in starch saccharification (Dicko *et al*., 2006). However, low β-amylase activity of sorghum grains leads to limited fermentable extract (Agu and Palmer, 1998). Thus sorghum processing for bioethanol production usually exploits the addition of commercial enzymes. du Preez *et al*. (1985) used sorghum grains for bioethanol production via a dual-enzymatic process. The enzymes used were Termamyl 120L and AMG 200L (du Preez *et al*., 1985). Sorghum hydrolyzate was obtained by liquefaction and saccharification of flaked sorghum grains in a bioreactor. For liquefaction, Termamyl 120L, which is an α-amylase was added at a temperature of 60°C for 60 minutes. For saccharification, AMG 200L, an amyloglucosidase was added at a temperature of 65°C for 120 minutes (du Preez *et al*., 1985). The hydrolyzate was fermented using *S.cerevisiae.* An ethanol concentration of 12% (v/v) was obtained (du Preez *et al*., 1985).

In another study, Suresh *et al*. (1999) showed that bioethanol production is possible using damaged sorghum by saccharification of raw starch and fermentation using α-

amylase from *Bacillus subtilis* and *S. cerevisiae*. Damaged sorghum produced 3.5% (v/v) ethanol. This is less than 5% (v/v) ethanol produced from non-damaged sorghum. Although, ethanol production is relatively decreased, the ethanol can still be used for industrial purposes and damaged grains are cheaper (Suresh *et al*., 1999).

In addition, bioethanol was produced by simultaneous saccharification and fermentation (SSF) in a bioreactor using mixed microbial cultures (Mamma *et al*., 1996). Sweet sorghum is composed of soluble and insoluble carbohydrates. The soluble carbohydrates such as glucose and sucrose are converted rapidly to ethanol. The insoluble carbohydrates such as cellulose and hemicellulose are of great interest for bioethanol production (Mamma *et al*., 1996). SSF is useful for the conversion of these insoluble carbohydrates into ethanol. A mixed microbial culture of *Fusarium oxysporum* and *S.cerevisiae* was used for SSF. *F.oxysporum* is involved in enzyme production necessary for saccharification of cellulose and hemicellulose. When grown aerobically, *F.oxysporum* produces cellulolytic and hemicellulolytic enzymes necessary for the hydrolysis of the insoluble carbohydrates (Mamma *et al*., 1996). The addition of *S.cerevisiae* then converts the available sugars to ethanol (Mamma *et al*., 1996). An ethanol concentration of 3.5-4.9% (w/v) is obtained using sorghum stalks (Mamma *et al*., 1996).

(2.2) Methodology

2.2.1. Experimental Design

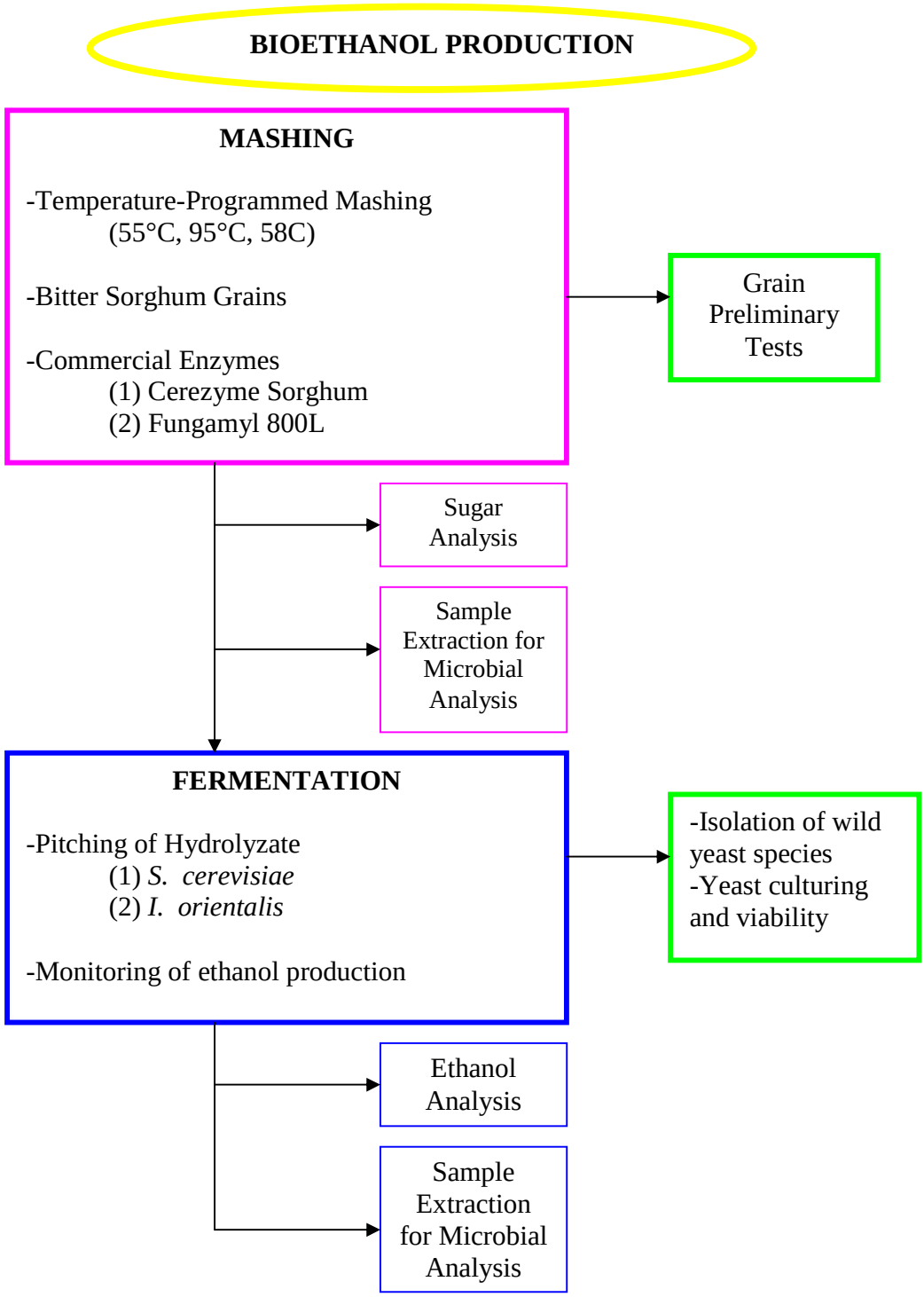


Fig 2.2 Schematic Diagram representing an outline of the Experimental Design

2.2.2. Experimental Approach

2.2.2.1. Grain Preliminary Tests

The Red Swazi variety of sorghum grains, kindly supplied by TigerBrands Ltd, South Africa, was used. Prior to processing, whole sorghum grains were tested for the presence of a pigmented testa by the Chlorox Bleach test as described by Waniska *et al*. (1992). One-hundred whole grains, in 50mL beakers were immersed in Chlorox Bleaching Reagent (5g NaOH, 100mL 3.5% commercial bleach). The beakers were then covered with foil and incubated at room temperature (20°C-30°C) for 20 minutes, whilst the contents of the beaker were swirled at 5 minute intervals. Post-incubation, the grains were emptied into a strainer, rinsed with tap water and blotted dry using paper towel. The test was carried out in triplicate. The number of black grains was counted and the percentage of tannin sorghum was determined (Waniska *et al*., 1992).

2.2.2.2. Isolation of a Wild Yeast Species

Sorghum-sour porridge was used to isolate a wild yeast species for fermentation. A loop-full of sorghum-sour porridge was streak plate onto Potato Dextrose Agar (PDA, Merck). Plates were incubated at 30°C for 48 hours. Post-incubation, morphologically different colonies were picked and grown in Yeast Peptone Dextrose (YPD-10g/L yeast extract, 20g/L peptone, 20g/L glucose) broth culture. The broth culture was incubated at 30°C for 24 hours with shaking at 180rpm (Rotary Shaker). Broth culture was used for purity streak plates onto PDA. Pure colonies from PDA were used for identification by the Gram Stain method, Polymerase Chain Reaction (PCR) and Automated Sequencing. The Gram Stain method was performed as described by Gerhardt *et al*. (1985). PCR and

Sequencing was performed by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. For PCR, ITS 1 and ITS 4 primers were used for amplification and ITS 4 primer was used for sequencing of the PCR product. Basic Local Alignment Search Tool (BLAST) was used to analyze the sequence, and identify unknown isolates.

Following identification procedures, a pure wild yeast isolate was preserved at -70°C using 50% glycerol, until further culturing.

2.2.2.3. Yeast Culturing and Viability

(a) Yeast Culturing

S.cerevisiae and a wild yeast isolate were cultured for fermentation. *S.cerevisiae* (dry brewers yeast) was obtained from National Food Products (Emmarentia, Johannesburg). The wild yeast species was isolated as previously described **(2.2.2.2).** Both yeast species were cultured using YPD broth culture. For the wild yeast isolate, a single glycerol stock was added to 50mL of YPD broth culture, which was the starting culture volume. For *S.cerevisiae* one packet of dry brewers yeast was added to 200ml of YPD broth culture and pure colonies were obtained by two successive streak plates onto Malt Extract Agar (MEA, Merck). The Gram Stain method, PCR and Automated Sequencing were performed as previously described **(2.2.2.2)**, to confirm for a pure *S.cerevisiae* isolate. Following identification, the pure isolate was preserved in 50% glycerol at -70°C. This glycerol stock was added to an initial culturing volume of 50mL YPD broth. The inoculum flasks were incubated at 30°C for 24 hours with shaking at 220rpm. Every 24 hours, cell number was increased by sub-culturing at 10%.

Culturing volumes for scale-up of inoculum were as follows:-

50mL ➡ 200mL ➡ 250mL ➡ 500mL ➡ 750mL ➡ 1L ➡ 1.5L

Incubation conditions were maintained for all culturing volumes. Post-culturing, the yeast cells were re-suspended in 85% (v/v) saline solution. The 1.5L broth culture was aliquot into 50mL NUNC tubes (Greiner Bio, LASEC) and centrifuged (Biofuge) at 7,500rpm for 5 minutes. The supernatant was discarded and the pellet was re-suspended in 20mL of 85% saline by gently vortexing.

(b) Yeast Viability

Yeast viability was determined, post-culturing, by dilution series and plate counts. The dilution series was carried out using Buffered Peptone Water (BPW, Merck), as the diluent. Plate counts were determined using MEA and PDA for *S.cerevisiae* and the wild yeast species, respectively. Tenfold serial dilutions (10^{-1}-10^{-6}) were carried out in duplicate using the 1.5L broth culture. From each appropriate dilution, 0.1mL of the diluted suspension was spread plate onto MEA and PDA. Plates were incubated at 30°C for 48 hours. Post-incubation, plates showing between 30-300 colonies were selected for enumeration and the pitching rate was determined according to the following equation:-

$$\text{Colony Forming Units/mL} = \frac{\text{Number of Colonies X Dilution Factor}}{\text{Volume}}$$

2.2.2.4. Sorghum Processing for Bioethanol Production

Sorghum processing was carried out using the Wits Microbrewery Plant (School of Chemical and Metallurgical Engineering, Wits University), represented in Fig 2.3.



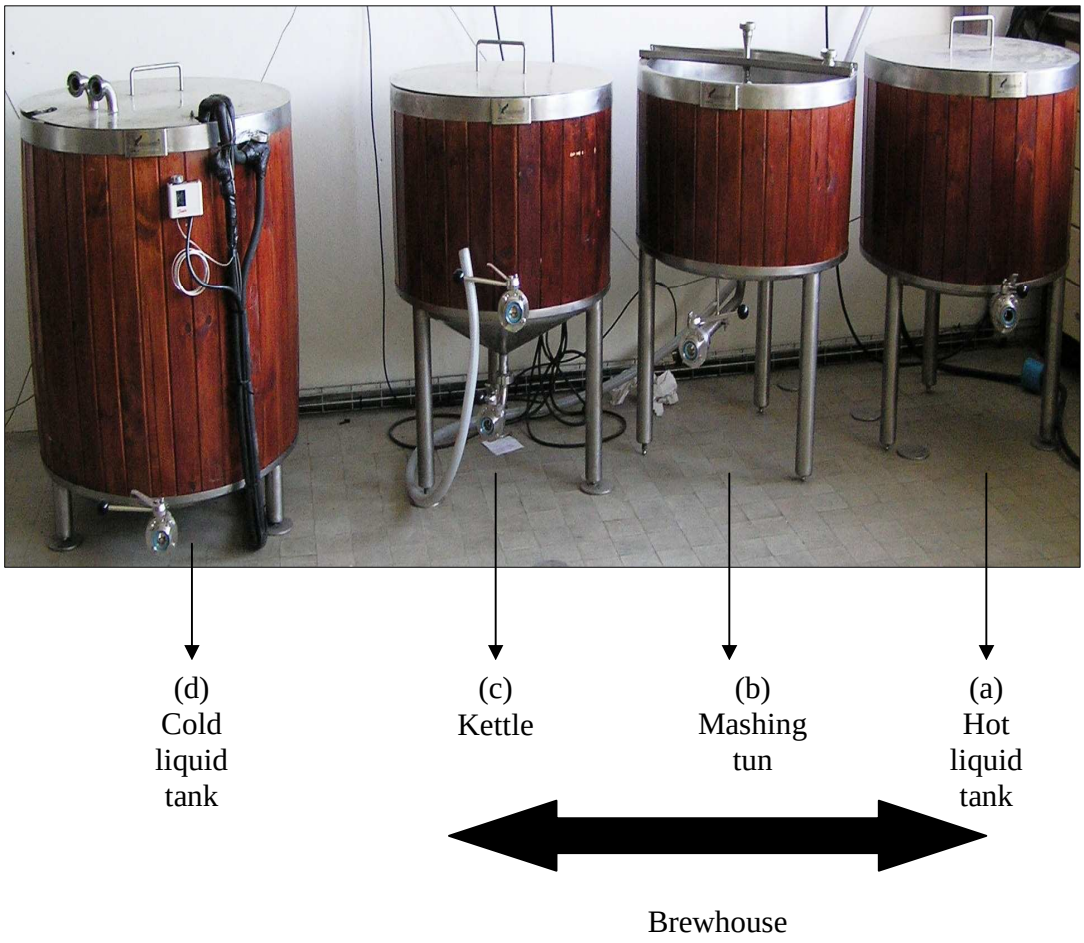
Fig 2.3. The Wits Microbrewery Plant-designed and supplied by Falcon Engineering (Pty) Ltd, South Africa.

Prior to processing, the un-malted sorghum grains (Fig 2.4a) were dry milled into a coarse powder (Fig 2.4b) using a 2-roller laboratory mill. Sorghum processing involved:- (a) mashing and (b) fermentation.

(a)  (b) 

Figs 2.4a-b. Red Swazi Variety of Sorghum Grains. (a) Un-malted Sorghum Grains. (b) Milled Sorghum Grains.

(a) Mashing



Figs 2.5a-d. Brewhouse and Cold Liquid Tank of the Wits Microbrewery Plant.

The mashing procedure was performed using the brewhouse (Figs 2.5a-c). During mashing, the starch of the grain was enzymatically hydrolyzed via a two-step enzyme process using commercial exogenous enzymes. The commercial enzymes were kindly donated by Novozymes SA (Pty) Ltd, Marlboro, South Africa. Information regarding the enzymes is described in Table 2.3.

Table 2.3. Summary of commercial enzymes from Novozymes

Product Name	Physical Form	Enzyme Activity	Origin	Mashing Function
Cerezyme Sorghum	Solid	α-amylase β-glucanase Protease	Genetically modified sorghum	Liquefaction Saccharification
Fungamyl 800L	Liquid	α-amylase	*Aspergillus oryzae*	Saccharification

Mashing was carried out in the kettle (Fig 2.5c), which was suitable for a temperature-programmed mashing regime. The sieve from the mash tun (Fig 2.5b) was transferred to the kettle for hydrolyzate extraction. Brewing liquor was pretreated with 5mL phosphoric acid and heated to approximately 65°C in the hot liquid tank (Fig 2.5a). The heated liquor was then pumped into the kettle (Fig 2.5c). Thirty kilograms (30kg) of milled sorghum grains (stored at room temperature), supplemented with 20g of calcium chloride and 20g of sodium chloride was mixed with the heated brewing liquor. The mashing regime was as follows:- at 55°C 150g of Cerezyme Sorghum (50KNU-S/g; 6FBG/g; 0.2AU-A/g) was added to the mixture for 30 minutes, followed by boiling at 95°C for 60 minutes. Post-boiling, the mash was cooled, at 58°C 100mL of Fungamyl 800L (800FAU-F/g) was added and mashing was continued for 60 minutes. Post-mashing, the mixture was boiled for 60 minutes, to inactivate the enzymes. The pH was in the range of 4.5 to 5.5 during mashing, which was optimum for the functionality of both enzymes. For the control, hydrolysis was performed without the addition of the exogenous enzymes. Mashing conditions were recommended by Novozymes (enzyme manufacturers).

Following mashing, first-runnings, sparging and second runnings was performed. First runnings, involved re-circulating the hydrolyzate from the bottom outlet valve of the kettle to extract the hydrolyzate from the grains. The re-circulated hydrolyzate was then

transferred into the mash tun (Fig 2.5b) for short-term shortage whilst simultaneous sparging and second runnings was performed. For sparging, brewing liquor at 65°C was poured on to the grain filter bed to further extract the remaining hydrolyzate from the spent grains. As sparging continues, second runnings hydrolyzate was collected from the bottom outlet valve of the kettle and added to the mash tun. From the mash tun the hydrolyzate was pumped through the heat exchanger and into the fermenter. Water from the cold liquid tank (Fig 2.5d) was passed through the heat exchanger for cooling by heat exchange. Throughout the mashing procedure, the sorghum slurry was manually agitated by mixing the slurry with a wooden stirrer.

(b) Fermentation



Fig 2.6. Conical Fermentation Tank (100L) of the Wits Microbrewery Plant.

Fermentation was carried out in a 100L conical fermenter (Fig 2.6). The hydrolyzate, consisting of varying concentrations of sugars was inoculated with 500mL of the yeast-saline suspension and subjected to aerobic fermentation for two days, followed by anaerobic fermentation by either *S.cerevisiae* or the wild yeast isolate. Ethanol production was monitored at 24 hour intervals, until maximum ethanol concentration of was reached.

2.2.2.5. Analytical Methods

(a) Analyses of fermentable carbohydrates

High Performance Liquid Chromatography (HPLC) was used to analyze the fermentable sugars obtained by enzymatic and non-enzymatic hydrolysis.

(i) Apparatus

The HPLC apparatus consisted of:- Zorbax Carbohydrate Column (4.6mmIDX150mm.; 5um); Refractive Index Detector (HP 1100); Guard Column (4.6mmIDX12.55mm) and a 100uL Automatic Injector. The Chemstation Software was used for data analysis.

(ii) Chemicals and Standards

All chemicals used for HPLC were of analytical reagent grade (Sigma Aldrich) and solutions were prepared using deionized water. Carbohydrate standards (Fig 2.7) used for construction of the calibration curve was:- fructose (3.2mg/mL); glucose (12mg/mL); sucrose (12mg/mL); maltose (20mg/mL); and lactose (20mg/mL). The sample injection volume was 1uL.

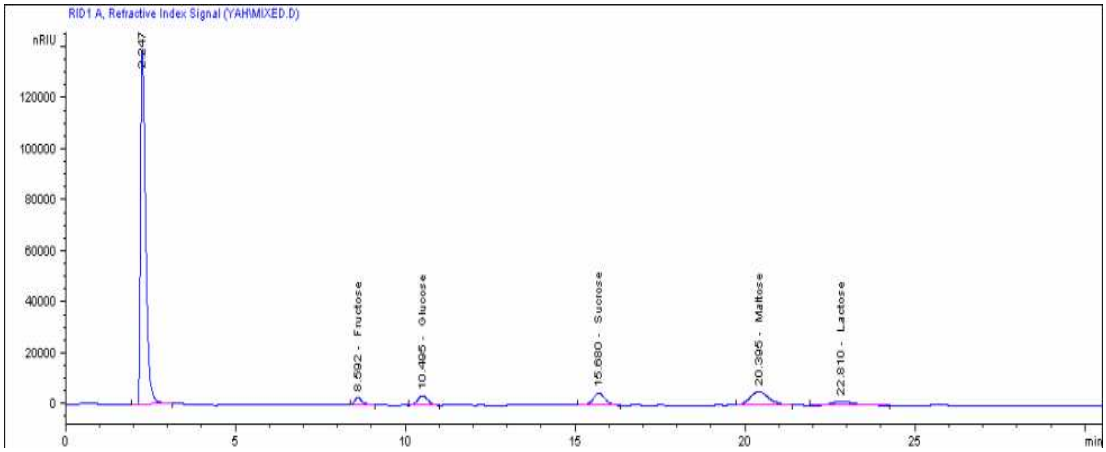


Fig 2.7. HPLC Chromatogram of five carbohydrate standards. Peak1: Fructose, Peak 2: Glucose, Peak 3: Sucrose, Peak 4: Maltose, Peak 5: Lactose.

(iii) Samples and Column Conditions

Post-mashing samples were filtered using a 0.22 micron filter. Prior to sugar analysis of the sorghum hydrolyzate by HPLC, the system was purged with isopropanol (75:25, v/v) and standards were run. The column operations were as follows:- temperature 30°C, flow rate 1.4mL/min, sample injection volume 20uL; mobile phase 75/25 Acetonitrile/Water.

(b) Ethanol Analyses

Specific gravity and alcohol content was monitored at 24h intervals, using samples extracted from the bottom outlet valve of the fermenter. Post-fermentation samples were centrifuged (Biofuge) to remove yeast particles. Five hundred milliliters (500mL) of the supernatant was used for the reading. Specific gravity was determined using a Hydrometer, as an indicator for the start of ethanol production and to detect when maximum ethanol was reached. The ethanol content was determined using an Alkoholometer according to the Gay-Lussac % (v/v). Ethanol analyses were based on

constructing a standard curve represented in Fig 2.8. Standard mixtures of 5% (v/v), 10% (v/v), 15% (v/v), and 20% (v/v) of absolute ethanol were prepared in deionized water.

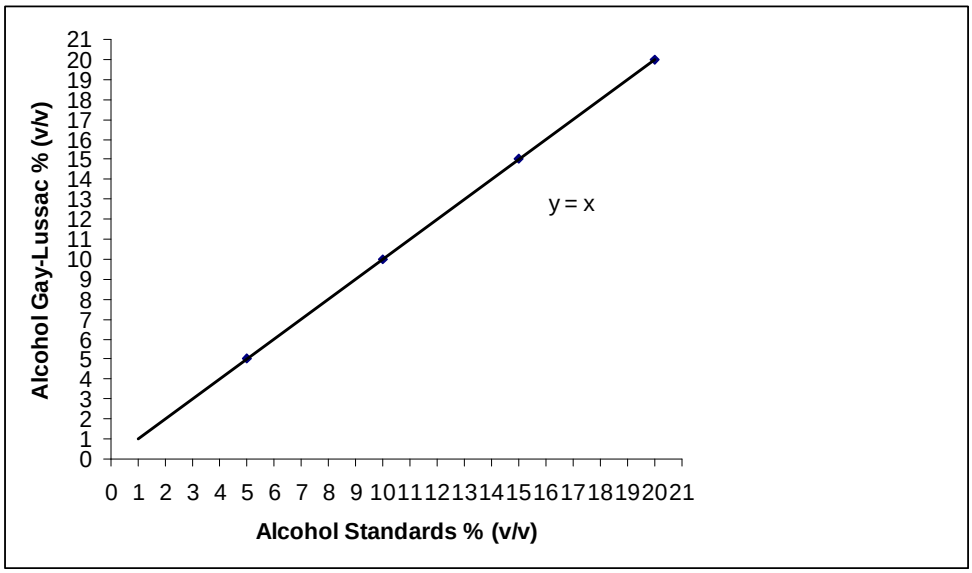


Fig 2.8. The Alcohol Gay-Lussac % (v/v) of standard mixtures of alcohol ranging from 5-20% (v/v).

To measure the ethanol content with the Alkoholometer, pure distillate was not used. In order to validate the readings obtained from the Alkoholometer, sugar standards were used. Sugar standards of five carbohydrates were prepared. The carbohydrates were:- glucose, fructose, maltose, sucrose, and lactose. Standard preparations were as follows:- for each of the five sugars, a fixed volume of alcohol (5%, v/v) was aliquot into 5g, 10g, 15g, and 20g of sugars and dissolved with deionized water to a final volume of 500mL. The alcohol percentage was then measured for each concentration of the sugars using the Alkoholometer.

(2.3) Results and Discussion

2.3.1. Grain Preliminary Tests

The results of the Chlorox Bleach test was positive, as whole grains of the Red Swazi variety of sorghum turned black after immersion of the grains in Chlorox Bleaching reagent (Waniska *et al*., 1992). The average number of grains that turned black divided by 300 grains indicated that the percentage of tannin sorghum was 98%. Therefore, this particular variety of sorghum consists of a pigmented testa and is a condensed tannin variety. This is based on the fact that treating sorghum grains with bleaching reagent dissolves the pericarp of the grain and exposes the pigmented testa. Sorghum varieties with a pigmented testa, usually turn black and are condensed tannin varieties. Whereas, grains lacking a testa, are brown or white and are condensed non-tannin varieties (Waniska *et al*., 1992).

2.3.2. Identification of Yeast Isolates

A “putative” wild yeast species was isolated from sorghum-sour porridge. Optimal growth conditions showed the appearance of various morphologically different colonies on agar plates. Macroscopic morphological characteristics of purity streak plates revealed that predominant colonies on PDA were white in colour with undulate margins (Fig 2.9a). Microscopic examination via the Grams Reaction showed large, cylindrical cells which stained gram positive (Fig 2.9b). To ascertain that the “putative” isolate was a wild yeast species, PCR amplification of the internal spacer region (ITS) was carried out. Positive amplicons were sequenced. The BLAST Software Programme (Altschul *et al*., 1990) was used to analyze the sequence. BLAST analysis revealed 100% sequence

identity to *Issatchenkia orientalis. I.orientalis* is a non-*Saccharomyces* wild yeast which was previously isolated from *togwa* (Mugula *et al*., 2003). *Togwa* is a Tanzanian traditional fermented food prepared using sorghum, maize, cassava and millet (Mugula *et al*., 2003).

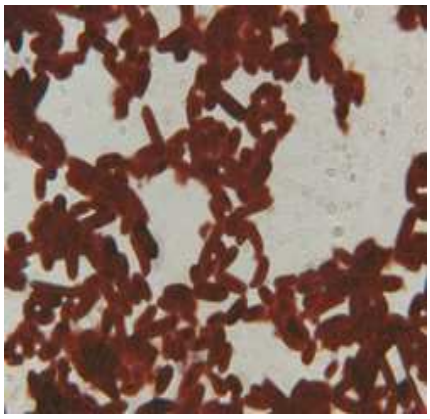
(a)  (b) 

Fig 2.9a-b. (a) Colonies of wild yeast isolate on PDA. (b) Grams stain of wild yeast isolate.

Furthermore, PCR amplification and sequencing of *S.cerevisiae* (dry brewers yeast) was performed. BLAST analysis confirmed 100% sequence identity to *S.cerevisiae* strain NRRL Y2084. For the purpose of the report, *S.cerevisiae* strain NRRL Y2084 will be referred to as *S.cerevisiae.*

2.3.3. Yeast Pitching Rate

For culturing of each yeast species, YPD broth was prepared and inoculated with pure glycerol stocks of the isolates. The pitching rate of the *S.cerevisiae* suspension was ~10^8 cells/mL and the *I.orientalis* suspension was ~10^6 cells/mL. The population number of *I.orientalis* was lower compared to *S.cerevisiae.* However, growth conditions,

culturing volumes and growth medium allowed for high aerobic counts, sufficient for fermentation.

2.3.4. Sorghum Processing for Bioethanol Production

(a) Hydrolysis of Starch

Of the various cereal grains that can be used for bioethanol production, bitter sorghum grains were chosen as starting material for this investigation as it is extensively grown in South Africa and the starch content of sorghum ranks with barley.

The dual-enzyme combination of Cerezyme Sorghum and Fungamyl 800L was used for the hydrolysis of sorghum grain starch into fermentable sugars. The enzymes were chosen as this particular combination for hydrolysis of bitter sorghum grains to produce bioethanol has not been previously investigated. Cerezyme Sorghum is extracted from genetically modified sorghum plants and thus adapted to breakdown sorghum specific starch. Furthermore, it is a mixture of enzymes with broad-range activity (Table 2.3). Fungamyl 800L, an α-amylase (Table 2.3) serves as a saccharifying enzyme using the liquefied extract as a substrate. Both enzymes posses α-amylase activity, as sorghum α-amylase activity is generally lower than other grains (Taylor et al., 2006).

HPLC analysis proved that these commercial enzymes can produce sugars from raw or un-malted bitter sorghum grains. The results of HPLC revealed three major carbohydrates (Figs 2.10 and 2.11). Post-mashing profiles of the carbohydrates are shown in the chromatograms (Figs 2.10 and 2.11).

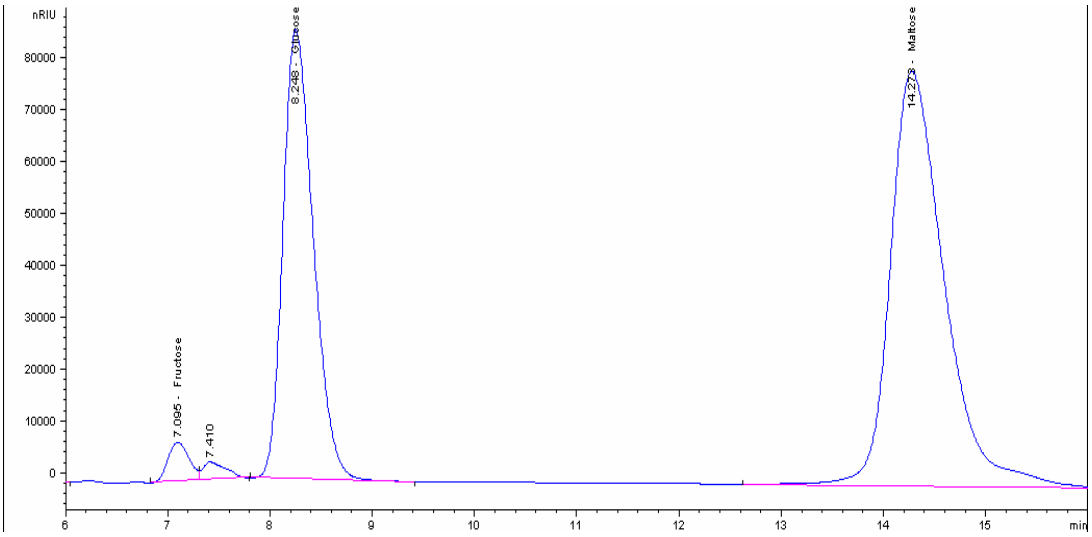


Fig 2.10. HPLC Chromatogram of the post-mashing sample with the addition of exogenous enzymes of sorghum processing 1 (SP1). Peak identification and retention times are indicated on the chromatogram according to the order of elution from the Zorbax Carbohydrate Column. Peak 1: Fructose, Peak 2: Unknown, Peak 3: Glucose, Peak 4: Maltose.

Chromatograms of sorghum processing 1 (SP1) and sorghum and sorghum processing 2 (SP2) were identical and only the SP1 chromatogram is displayed in this report. Fructose, glucose and maltose show single peaks (Fig 2.10). Peak areas detected by the refractive index detector (RID) are almost equal for glucose and maltose (Fig 2.10). The peak area of fructose is smaller compared to glucose and maltose and the unknown peak (peak 2) elutes close to the fructose peak (Fig 2.10).

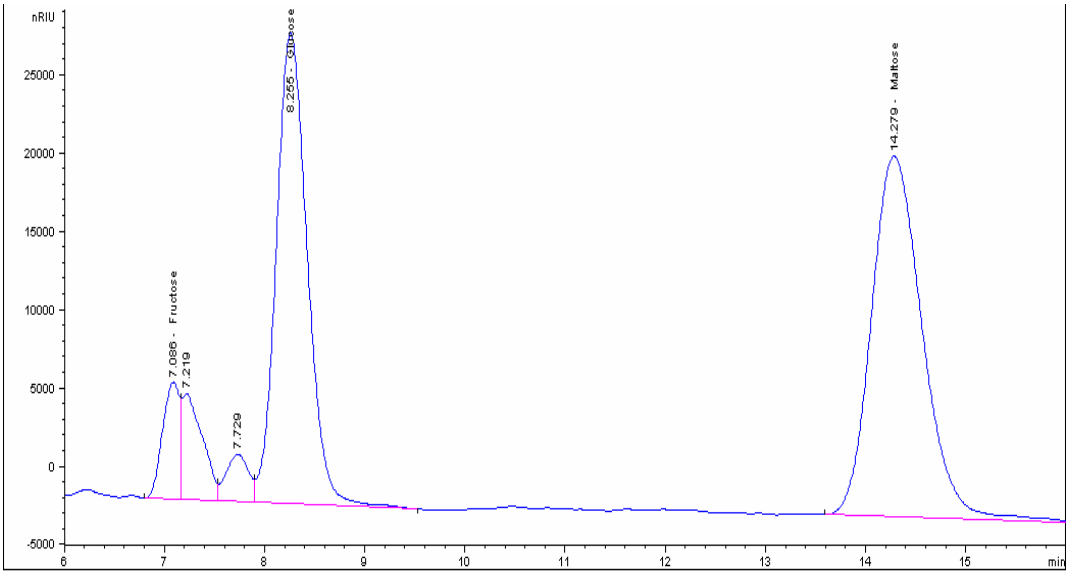


Fig 2.11. HPLC Chromatogram of the post- mashing sample without the addition of exogenous enzymes (control). Peak identification and retention times are indicated on the chromatogram according to the order of elution form the Zorbax Carbohydrate Column. Peak 1: Fructose. Peak 2: Unknown, Peak 3: Unknown, Peak 4: Glucose, Peak 5: Maltose.

For the control, single peaks were observed for fructose, glucose, maltose and unknown peak 3 (Fig 2.11). Once again, the peak area of fructose is smaller and unknown peak 2 elutes with fructose (Fig 2.11).

The unknown peaks may be maltotriose (Muguala *et al*., 2003b), raffinose (Anglani, 1998) or possibly stachyose, which is present in small quantities in sorghum (Subramanian *et al*., 1980). The co-elution between fructose and unknown peak 2 (Figs 2.10 and 2.11) could be due to similar retention times of these compounds. To improve identification of the unknown peaks a different detector, such as Mass Spectrometry can

be used. Fructose, glucose and sucrose are the dominant sugar components of sorghum (Subramanian *et al*., 1980; Anglani, 1998). Other sugars such as maltose and raffinose are usually <1.0% of the total sugar content (Anglani, 1998). In this study, a single sucrose peak was not detected. A possible reason could be the conversion of sucrose into glucose and fructose based on the dual α-amylase activity of the exogenous enzymes. In contrast, there could be a hindrance in α-amylase activity caused by the presence of tannins (Mullins and NeSmith, 1988), preventing initial breakdown of sucrose from starch granules. As detected by the Chlorox Bleach test, the Red Swazi variety of sorghum grains used in this investigation is a condensed tannin variety. Tannins are phenolic compounds that have an affinity for various sites on proteins (Mullins and Lee, 1991). Enzymes, which are essentially proteins, are affected by tannins as these compounds bind and form irreversible complexes, causing a reduction in the activity of the enzymes (Glennie, 1983; Mullins and NeSmith, 1988; Mullins and Lee, 1991). In theory, reduced enzyme activity is due to the aromatic rings of the tannins and the aromatic or proline residues of the enzymes binding to form non-polar hydrophobic aggregates (Mullins and Lee, 1991; Duodu et al., 2003). The effect of tannins on the exogenous enzymatic activity of Cerezyme Sorghum and Fungamyl 800L will require verification by enzyme-binding assays. Other reports have proposed that pre-treatment of the grains, prior to the addition of the exogenous enzymes with formaldehyde will complex with the tannins and in turn prevent tannin-protein complexes (Mullins and NeSmith, 1988; Taylor *et al*., 2006). With regards to this study, formaldehyde is a health and safety concern for the microbrewery plant. The mashing experiments can be further optimized by:- gelatinization; pre-treatment with other chemicals such as sodium or

ammonium hydroxide (Waichungo and Holt, 1995; Taylor *et al*., 2006); or increasing the concentration of the enzymes or increasing the mashing times.

The concentrations of the carbohydrates were quantified by the HPLC-RID and is presented in Table 2.4

Table 2.4. Quantitative Analysis of Carbohydrates as detected by HPLC.

Carbohydrates	**Exogenous Enzymatic Hydrolysis-Sorghum Processing 1 mg/mL**	**Exogenous Enzymatic Hydrolysis-Sorghum Processing 2 mg/mL**	**Hydrolysis without the addition of exogenous enzymes (Control) mg/mL**
Fructose	9.0	9.1	7.6
Glucose	16.2	17.1	6.2
Maltose	30.0	32.0	8.8
Total	**55.2**	**58.2**	**22.6**

An observation of the quantitative carbohydrate values showed that the total carbohydrate content of 55.2mg/mL (SP1) and 58.2mg/mL (SP2) was higher compared to hydrolysis without exogenous enzymes, which resulted in a total carbohydrate content of 22.6mg/mL (Table 2.4). The low extract yield of the control was expected as it was suggested that un-malted sorghum grains do not exhibit β-amylase activity (Agu and Palmer, 1998). Beta-amylase activity is activated during malting (Agu and Palmer, 1998). Without the use of exogenous enzymes, the combined diastatic power of α- and β-amylase is necessary for sufficient extract (Agu and Palmer, 1998). Carbohydrate concentrations did not vary much between SP1 and SP2 as mashing conditions were constant, as per manufacturers instructions (Novozymes). Although the carbohydrate content is unchanged, sorghum grain starch conversion increases with the addition of

exogenous enzymes (Table 2.4). Cerezyme Sorghum used in this study had α-amylase and β-glucanase activity, this is an advantage as commercial enzymes of this combination are required for increased recovery of sugar extract from sorghum raw materials (Omidiji and Okpuzor, 2002). Maltose is the predominant sugar (Table 2.4). This was unexpected as other results have proposed that sorghum extracts usually have high glucose yields and low maltose yields (Agu and Palmer, 1998). The high maltose concentrations obtained may be due to an obstruction in α-amylase activity, blocking further break down into its monosaccharide components. Enzyme activity is possibly affected by tannins, as discussed earlier. In this report, the total sugar content is lower compared to a previous report where 130g/L of sugars was obtained using bitter sorghum grains (du Preez *et al*., 1985). The investigation by du Preez *et al*. (1985) involved hydrolysis of tannin sorghum with the use of a bacterial α-amylase, ie. Termamyl 120L and a fungal amyloglucosidase, ie. AMG 200L. In another related study, damaged sorghum was processed by α-amylase extracted from *Bacillus subtilis* (Suresh *et al*., 1999). The sugar content produced was 63.50g/L (Suresh *et al*., 1999). This value is similar to the concentration obtained in this study. Hence it is observed that, different types of commercial enzymes produce different concentrations of sugars.

(b) Evaluation of Bioethanol Production

One of the main problems faced today is the depletion of fossil fuels. A common solution for fuel limitations is the production of bioethanol from renewable energy resources via fermentation. For the purpose of this investigation, fermentation experiments were

conducted separately with *S.cerevisiae* and *I.orientalis*, using sorghum hydrolyzate for bioethanol production.

Sorghum hydrolyzate with a total sugar content of 55.2mg/mL was fermented using *S.cerevisiae*, referred to as SP1. For SP2, the total sugar content was 58.2mg/mL and was fermented with *I.orientalis*. The total sugar content of the control was 22.6mg/mL and fermentation was performed using *S.cerevisiae*. The results of fermentation are presented in Fig 2.12.

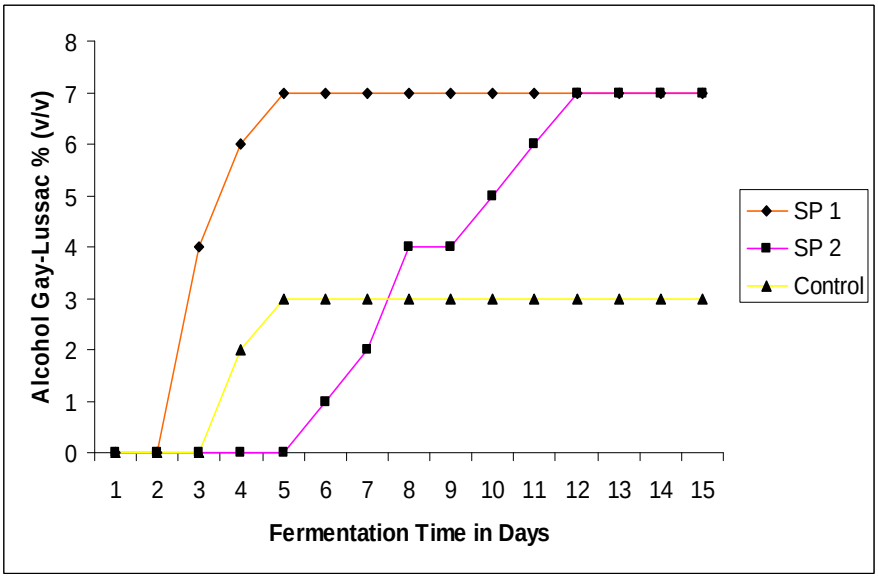


Fig 2.12. Maximum ethanol production by *S.cerevisiae* and *I.orientalis* of hydrolysed starch obtained from bitter sorghum grains and exogenous enzymes.

SP 1: indicates fermentation by *S.cerevisiae* of enzymatically hydrolysed starch.

SP 2: indicates fermentation by *I.orientalis* of enzymatically hydrolysed starch.

Control: fermentation by *S.cerevisiae* of hydrolyzate obtained without the addition of exogenous enzymes.

It is evident from Fig 2.12 that the yeasts used are capable of growth and fermentation under the fermentation parameters of:- 19°C, pH~4.0 and pitching rate of ~10^8 cells/mL for *S.cerevisiae* and ~10^6 cells/mL for *I.oreintalis.* The patterns of fermentation of the yeast species are different. *S.cerevisiae* is capable of utilizing the soluble carbohydrates within 5 days (Fig 2.12). Whereas, *I.orientalis* is a slow fermenting yeast reaching maximum ethanol at day 12 (Fig 2.12). A possible reason for the delayed effect of *I.orientalis* is that this particular yeast species requires a longer lag period to the fermentation environment. To increase the rate of fermentation, co-inoculation of *I.orientalis* with other yeast species is a possibility. Furthermore, *S.cerevisiae* had a distinct advantage as the concentration of yeast cells at point of pitching was higher than that of *I.orientalis.*

During fermentation, specific gravity (SG) and the alcohol content was measured using a hydrometer and an Alkoholometer, respectively. The readings were taken at room temperature at the point where the sample liquid intersected the instrument. The hydrometer readings were an indication of the progression of fermentation. Prior to yeast pitching, the SG of the sorghum extract from SP1 and SP2 was ~1.050. The SG of the control was ~1.020. At day 3, day 4 and day 6 for SP1, the control and SP2, respectively, a decrease in SG was observed. This is a clear indication that alcohol is being produced. The alcohol content, expressed as Gay-Lussac % (v/v) (Fig 2.12), was measured with an Alkoholometer ranging from 0-100%. For the alcohol readings, pure distillate was not used. Results were validated based on sugar standards as described in **2.2.2.5b**. This showed that the readings were unaffected by various sugar concentrations. The readings presented in this study are a true account of the values. The fermentation start time in

days for *S.cerevisiae* is day 3 and produced an initial alcohol concentration of 4% (v/v). (Fig 2.12). *I.orientalis* fermentation start time in days is day 6 and produced 1% (v/v) of alcohol (Fig 2.12). Fermentation start time, for the control, is at day 4 with a 2% (v/v) concentration of alcohol (Fig 2.12). As SG decreases, alcohol concentration increases. Observations from Fig 2.12 indicate that the maximum yield of exogenous enzymatically hydrolyzed starch (SP1 and SP2) is 7% (v/v). For the control (hydrolysis without exogenous enzymes), 3% (v/v) is the maximum ethanol yield. Maximum ethanol readings are based on SG. When SG reaches 1.000, steady state is reached, fermentation is complete and the maximum level of alcohol from the available sugar is produced. The amount of bioethanol obtained in this investigation is economically low, as du Preez *et al*. (1985) suggest that 8-9% (v/v) is acceptable for distillation. In comparison to other studies, similar results were reported by Suresh *et al*. (1999), as 5% (v/v) of bioethanol was produced with the use of sorghum flour via simultaneous saccharification and fermentation (SSF) (Suresh *et al*., 1999). Mamma *et al*. (1996) reported that 3.5-4.9% (v/v) of bioethanol was produced via SSF using sweet sorghum stalks. However, du Preez *et al*. (1985) showed an ethanol concentration of 12% (v/v) was produced from a condensed tannin variety. Thus, bioethanol yield varies depending on the type of raw materials and processing conditions used.

There are several contributing factors likely to affect the yield of bioethanol. The lower free amino nitrogen (FAN) levels usually associated with sorghum processing (Ng`andwe *et al*., 2008) may be a nutrient limiting factor. FAN is produced during mashing and originates from the breakdown of proteins present in the grains. It is utilized by yeasts as a growth supplement during fermentation and assists with the ease of “sugar

comsumption" (Yano *et al*., 2008). Even though the exogenous enzyme, Cerezyme Sorghum exhibits protease activity, the presence of tannins could affect the conversion of the grain proteins. Duodo *et al*. (2003) proposed that condensed tannin varieties of sorghum posses a tannin content that is capable of binding the proteins present in the grain to form insoluble precipitates (Duodo *et al*., 2003). The protein-tannin aggregates in turn decrease FAN levels which lead to nitrogen stress of yeast cells and lower fermentation rates (Mullins and Lee, 1991). It has been suggested that the fermentation rate can be improved by supplementation with nitrogen and protease enzymes (Mullins and Lee, 1991). Furthermore, polyphenol oxidase can be added to the fermentation tank to reduce tannins (Towo *et al*., 2006). In addition to tannins, phytic acid is a component of sorghum that affects protein breakdown and reduces trace minerals such as iron, which is important for yeast growth (Duodo *et al*., 2003; Towo *et al*., 2006). To overcome processing problems imposed by phytic acid, the fermentation procedure can be supplemented with phytase (Towo *et al*., 2006).

Fermentation experiments can be further optimized by the addition of external supplements (Mullins and Lee, 1991) to stimulate faster adaptation of the yeasts. Furthermore, fermentation can be improved by re-pitching of yeasts and installation of an agitator in the fermentation tank of the microbrewery plant. This will maintain the yeast in suspension and prolong the duration of fermentation which could increase the maximum attainable level of bioethanol.

(2.4) Conclusions

In conclusion, results of the present study shows:-

- Commercial exogenous enzymes, ie. Cerezyme Sorghum and Fungamyl 800L can convert starch from un-malted sorghum grain, of the Red Swazi variety, into sugars.
- There is a reduction in the level of carbohydrates when exogenous enzymes are not used during mashing of sorghum grains.
- The sorghum hydrolyzate is fermentable by *S.cerevisiae* strain NRRL Y2084 and *I.orientalis.*
- Maximum level of bioethanol produced from exogenous enzymatic hydrolyzate is 7% (v/v), whereas, 3% (v/v) of bioethanol is produced from hydrolyzate without exogenous enzyme addition.

(2.5) References

Aggarwal, N.K., Nigam, P., Singh, D. and Yadav, B.S. (2001). Process optimization for the production of sugar for the bioethanol industry from sorghum, a non-conventional source of starch. *Journal of Microbiology and Biotechnology.* **17:** 411-415.

Agu, R.C. and Palmer, G.H. (1998). A reassessment of sorghum for lager-beer brewing. *Bioresource Technology.* **66(3):** 253-261.

Altschul, S.F., Gish, W., Miller, W., Meyer, E.W. and Lipman, D.J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology.* **215(3):** 403-410.

Anglani, C. (1998). Sorghum Carbohydrates- A review. *Plant Foods for Human Nutrition.* **52:** 77-83.

Balat, M., Balat, H. and Cahide, O. (2008). Progress in Bioethanol Processing. *Progress in Energy and Combustion Science.* **34:** 551-573.

Bvochora, J.M. and Zvauya, R. (2001). Biochemical changes occurring during the application of high gravity fermentation technology to the brewing of Zimbabwean traditional opaque beer. *Process Biochemistry.* **37:** 365-370.

Demirbas, A. (2008). Biofuels sources, biofuel policy, biofuel economy and global biofuel projections. *Energy Conversion and Management.* **49:** 2106-2116.

Dicko, M.H., Gruppen, H., Traore, A.S., Voragen, A.G.J. and Berkel, W.J.H. (2006). Sorghum grain as human food in Africa: relevance of content of starch and amylase activities. *African Journal of Biotechnology.* **5(5):** 384-395.

Dlamini, N.R., Taylor, J.R.N. and Rooney, L.W. (2007). The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. *Food Chemistry.* **105:** 1412-1419.

Duodu, K.G., Taylor, J.R.N., Belton, P.S. and Hamaker, B.R. (2003). Factors affecting sorghum protein digestibility. *Journal of Cereal Science.* **38:** 117-131.

du Preez, J.C., de Jong, F., Botes, P.J. and Lategan, P.M. (1985). Fermentation Alcohol from Grain Sorghum Starch. *Biomass.* **8:** 101-117.

Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, N.A., Krieg, N.R. and Phillips, G.B. Manual Methods for General Microbiology. American Society for Microbiology. Washington, DC, 1981, pp: 21-33.

Glennie, C.W. (1983). Polyphenol changes in Sorghum Grain during Malting. *Journal of Agricultural Food Chemistry.* **31:** 1295-1299.

Kim, S. and Dale, B.E. (2004). Global potential bioethanol production from wasted crops and crop residues. *Biomass and Bioenergy.* **26:** 361-375.

Linde, M., Galbe, M. and Zacchi, G. (2008). Bioethanol production from non-starch residues in process streams from a dry-mill ethanol plant. *Bioresource Technology.* **99:** 6505-6511.

Mamma, D., Koullas, D., Fountoukidis, G., Kekos, D., Macris, B.J. and Koukious, E. (1996). Bioethanol from sweet sorghum: simultaneous saccharification and fermentation of carbohydrates by a mixed microbial culture. *Process Biochemistry.* **4:** 377-381.

Mojovic, L., Nikolic, S., Rakin, M. and Vukasinovic, M. (2006). Production of bioethanol from corn meal hydrolyzates. *Fuel.* **85:** 1750-1755.

Mugula, J.K., Nnko, S.A.M., Narvhus, J.A. and Sorhaug, T. (2003a). Microbiological and Fermentation Characteristics of *togwa,* a Tanzanian fermented food. *International Journal of Food Microbiology*. **80:** 187-199.

Mugula, J.K., Narvhus, J.A. and Sorhaug, T. (2003b). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of *togwa,* a Tanzanian fermented food. *International Journal of Food Microbiology*. **83:** 307-318.

Mullins. J.T. and NeSmith, C. (1988). Nitrogen Levels and Yeast Viability During Ethanol Fermentation of Grain Sorghum Containing Condensed Tannins. *Biomass.* **16:** 77-87.

Mullins, J.T. and Lee, J.H. (1991). Interactions of Tannins with Enzymes: A Potential Role in the Reduced Rate of Ethanol Fermentation from High-Tannin Biomass. *Biomass and Bioenergy.* **6(1):** 355-361.

Ng`andwe, C.C., Hall, A.N. and Taylor, J.R.N. (2008). Proteolysis of Sorghum Endosperm Proteins when Mashing with Raw Grain Plus Exogenous Protease and Potassium Metabisulphite. *Journal of the Institute of Brewing.* **114(4):** 343-348.

Odibo, F.J.C., Nwankwo, L.N. and Agu, R.C. (2002). Production of malt extract and beer from Nigerian Sorghum Varieties. *Process Biochemistry.* **37:** 851-855.

Omidiji, O. and Okpuzor, J.E. (2002). Enzymatic recovery of extract from cold trub derived from brewing with un-malted sorghum. (*Sorghum bicolor*). *Process Biochemistry*. **37:** 787-792.

Owuama, C.I. (1997). Sorghum: a cereal with lager beer brewing potential. *World Journal of Microbiology and Biotechnology*. **13:** 253-260.

Palmer, G.H. (1992). Sorghum-Food, Beverage and Brewing Potentials. *Process Biochemistry*. **27:** 145-153.

Pattison, T., Geornaras, I., and von Holy, A. (1998). Microbial populations associated with commercially produced South African sorghum beer as determined by conventional and petrifilm plating. *International Journal of Food Microbiology.* **43:** 115-122.

Sanchez, O.J. and Cardona, C.A. (2008). Trends in Biotechnological production of fuel ethanol from different feedstocks. *Bioresource Technology*. **99:** 5270-5295.

Subramanian, V., Jambunathan, R. and Suryaprakash, S. (1980). Note on the Soluble Sugars of Sorghum. *Cereal Chemistry*. **57(6):** 440-441.

Suresh, K., Kiransee, N. and Rao, L.V. (1999). Production of ethanol by raw starch hydrolysis and fermentation of damaged grains of wheat and sorghum. *Bioprocess Engineering*. **21:** 165-168.

Taylor, J.R.N., Schreuder, P.R.L., Joustra, S.M. and Nyandorah, M.G. (2003). Towards a Technical Academy in Sorghum Brewing. In: Proceedings of the ninth Scientific and Technical Convention. The Institute and Guild of Brewing, Africa Section, Victoria Falls, Zambia, pp: 33-36.

Taylor, J.R.N., Schober, T.J. and Bean, S.R. (2006). Novel food and non-food uses for sorghum and millets. *Journal of Cereal Science.* **44:** 252-271.

Towo, E., Matuschek, E. and Svanberg, U. (2006). Fermentation and enzyme treatment of tannin sorghum gruels: effects on phenolic compounds, phyate and in vitro accessible iron. *Food Chemistry*. **94:** 369-376.

Waichungo, W.W. and Holt, D.L. (1995). Use of Ammonium Hydroxide to Reduce the level of Assayable Tannin in High-Tannin Sorghum Grain. *Journal of Agricultural Food Chemistry*. **43:** 728-732.

Waniska, R.D., Hugo, L.F. and Rooney, L.W. (1992). Practical Methods to determine the presence of tannins in sorghum. *Journal of Applied Poultry Research.* **1:** 122-128.

Yano, M., Tsuda, H., Imai, T., Ogawa, Y. and Ohkochi, M. (2008). The Effect of Barley Adjuncts on Free Amino Nitrogen Contents in Wort. *Journal of the Institute of Brewing*. **114(3):** 230-238.

CHAPTER 3:
MICROBIOLOGICAL ANALYSIS

(3.1) Literature Review

3.1.1. Microorganisms Associated with Mashing and Fermentation Processes

End products of mashing and fermentation, sweet wort and beer respectively are considered to be unfavourable environments for the growth of microorganisms (MOs). This is due to:- high ethanol concentrations, acidic pH, low oxygen and high carbon dioxide content, minute amounts of sugars and antimicrobial properties of hop compounds (Sakamoto and Konings, 2003; Manzano *et al*., 2005). However, there are a range of MOs that are able to proliferate within these limited conditions. These include certain gram positive bacteria, gram negative bacteria and wild yeast species. They are referred to as spoilage MOs based on their ability to change the acidity and turbidity of fermentation by-products. In addition, the overall smell and flavours may be altered (Gram *et al*., 2002; Sakamoto and Konings, 2003). Spoilage MOs and the detection of these MOs will be discussed in relation to beer, as the processing of sorghum in this investigation is similar to the beer brewing technology.

3.1.2. Gram Positive Spoilage Bacteria

Gram positive bacterial species, grouped within the genera *Lactobacillus* and *Pediococcus* is hazardous to beer and the brewing industry (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). In Germany, during the 1980s lactobacilli and pediococci species caused approximately 58-88% spoilage of beer (Sakamoto and Konings, 2003).

3.1.2.1. The Genus *Lactobacillus*

A number of lactic acid bacterial species such as *Lactobacillus (Lb.) brevis, Lactobacillus lindneri, Lactobacillus curvatus, Lactobacillus casei, Lactobacillus buchneri, Lactobacillus coryneformis* and *Lactobacillus plantarum* are associated with beer spoilage (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). *Lb. brevis* and *Lb. linderi* have the highest spoilage potential (Sakamoto and Konings, 2003). *Lb. brevis* is a heterofermentative species which grows at an optimum temperature of 30°C and pH range of 4-6. *Lb. brevis* exerts it effect by the fermentation of dextrins and starch, and resistance to the antimicrobial activity of hop compounds (Sakamoto and Konings, 2003). *Lb. lindneri* is physiologically similar to *Lb. brevis* and exhibits resistance to hop compounds (Sakamoto and Konings, 2003). Optimal growth temperatures for *Lb. lindneri* is between 19-23°C (Sakamoto and Konings, 2003).

Lb. curvatus, Lb. casei, Lb. buchneri and *Lb. coryneformis* have a low spoilage potential in comparison to *Lb. brevis* and *Lb. lindneri* (Sakamoto and Konings, 2003). *Lb.casei* produces diacetyl. Diacetyl is the end product of the spontaneous conversion of α-acetolactate and contributes to a buttery-off flavour of beer (Priest and Campbell, 1987; Jespersen and Jakobsen, 1996, Sakamoto and Konings, 2003). Alpha-acetolactate is an intermediate compound released into the wort by brewing yeast during the synthesis of valine (Priest and Campbell, 1987). Additional lactic acid bacteria (LAB) detected in low numbers with spoilage capabilities include:- *Lb. brevismilis, Lb. malefermentans, Lb.*

parabuchneri and Lb. collinoides (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

3.1.2.2. The Genus *Pediococcus*

Pediococcus species associated with beer spoilage are *Pediococcus (P.) damnosus, Pediococcus acidilactici, Pediococcus dextrinicus, Pediococcus halophilus, Pediococcus inopinatus* and *Pediococcus parvulus* (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). These species can cause spoilage at various stages of the brewing process (Sakamoto and Konings, 2003).

P. damnosus and *P. inopinatus* are considered to be the most important for spoilage of beer. *P. damnosus* is a common inhabitant of beer but was not found as a contaminant of brewing raw materials (Priest and Campbell, 1987). *P. inopinatus* is associated with beer, wine, brewing yeasts and fermented foods such as milk and vegetables (Priest and Campbell, 1987). *Pediococcus* species are homofermentative bacteria. Spoilage is due to the formation of rope and large quantities of diacetyl (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). *P.inopinatus,* in particular requires an acidic pH of approximately 4.2, low concentration of ethanol and low concentration of hop compounds for spoilage (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

3.1.2.3. The Genus *Micrococcus*

The species *Micrococcus (M.) kristinae* is an additional gram positive beer spoilage MO belonging to the genus *Micrococcus* (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). *M. kristinae* grows under aerobic and anaerobic conditions. For spoilage,

M. kristinae requires a pH of 4.5 with reduced ethanol and hop compound concentrations, producing a fruity aroma (Jerspersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

3.1.3. Gram Negative Spoilage Bacteria

Bacterial species belonging to the genus *Pectinatus* and *Megasphaera* are the gram negative spoilage MOs of beer (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). Spoilage by these anaerobic bacteria is due to the production of hydrogen sulphide, acetic acid and dimethyl sulphide (Jespersen and Jakobsen, 1996). *Pectinatus* species are responsible for 20-30% of spoilage incidents, whilst *Megasphaera* species contributes to 3-7% spoilage (Sakamoto and Konings, 2003).

3.1.3.1. The Genus *Pectinatus*

Pectinatus cerevisiiphilus and *Pectinatus frisingensis* are the two main species of the genus *Pectinatus* (Sakamoto and Konings, 2003). These species are non-spore forming, rod-shaped bacteria with lateral flagella for motility (Sakamoto and Konings, 2003). Optimal growth conditions in beer are at a temperature of 32°C, pH between 4.3-4.6 and a 4.5-5% (w/w) ethanol concentration (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

Hydrogen sulphide, methyl mercaptan, acetic, proponic acids, acetoin and butyric acid are spoilage products formed during the growth of these bacteria in beer (Priest and Campbell, 1987; Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). This contributes to turbidity and a “rotten-egg smell” (Sakamoto and Konings, 2003).

3.1.3.2. The Genus *Megasphaera*

The species *Megasphaera cerevisiae* is a non-spore forming, cocci shaped bacterium.

M. cerevisiae grows at an optimal temperature of 28°C and a pH of 4.1. However, growth is inhibited at an ethanol concentration of ≥ 2.8% (w/v), although, it has been observed that growth can occur at ethanol concentrations between 3.8%-5.5% (w/v) (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). Spoilage potential of *M. cerevisiae* is similar to *Pectinatus* spp., where hydrogen sulphide production results in an undesirable odor. In addition, butyric acid, acetic acid and acetoin are produced (Sakamoto and Konings, 2003).

3.1.3.3. Additional Gram Negative Bacteria

In addition to the above mentioned gram negative spoilage bacteria **(3.1.3.1 & 3.1.3.2)**, other gram negative spoilage species include:- *Zymomonas (Z.) mobilis, Zymomonas raffinosivorans, Gluconobacter oxydans, Acetobacter aceti* and *Acetobacter pasteurianus* (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

Zymomonas spp. is phylogenetically related to *Pectinatus* spp. and thus growth conditions and spoilage potential is similar to that of the genus *Pectinatus* (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). Hydrogen sulphide, acetaldehyde, dimethyl sulphide and dimethyl disulphide are compounds produced by *Zymomonas* that contribute to spoilage (Priest and Campbell, 1987), resulting in a “rotten apple odour” (Priest and Campbell, 1987). *Z. mobilis* subsp. *mobilis* causes turbidity of beer as it produces high concentrations of hydrogen sulphide and acetaldehyde (Coton et al., 2008). Furthermore, *Z. mobilis* is an advantageous microorganism (MO). It is capable of surviving in ethanol concentrations of 10% (v/v) (Priest and Campbell, 1987) and has the

ability of fermenting glucose to form ethanol and carbon dioxide at a faster rate than yeast (Coton *et al*., 2008).

The aerobic, acetic acid bacteria, *Gluconobacter* and *Acetobacter*, causes a change in the flavour of the beer due to a "vinegary off-flavour" (Sakamoto and Konings, 2003). This is due to the conversion of ethanol into acetate (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003).

Furthermore, species belonging to the family Enterobacteriaceae is a threat to wort production during brewing (Jespersen and Jakobsen, 1996). *Klebsiella pneumoniae, Enterobacter aerogenes* and *Enterobacter agglomerans* are some of the species that contribute to spoilage of wort. However, these do not proliferate in beer (Jespersen and Jakobsen, 1996) and it is unknown if the flavour is affected by the presence of Enterobacteriaceae species (Priest and Campbell, 1987).

The species, *Hafnia protea* and *Enterobacter cloacae* of the Enterobacteriaceae family withstand fermentation and cause spoilage of the beer by producing dimethyl disulphide and fusel alcohols (Jespersen and Jakobsen, 1996).

3.1.4. Wild Yeasts in Beer Spoilage

Wild yeasts are yeast species that grow under processing (example-wort production) and packaging conditions of beer. Spoilage of beer can be caused by one wild yeast or 10^5-10^6 culture yeast. The wild yeast concentration results in the production of haze, turbidity, fatty acid and esters (van der Aa Kuhle and Jespersen, 1998). There are two

types of wild yeast, namely, *Saccharomyces* and non-*Saccharomyces* wild yeasts (Jespersen and Jakobsen, 1996).

Saccharomyces cerevisiae is responsible for the majority of beer spoilage by wild yeasts (Jespersen and Jakobsen, 1996). *S. cerevisiae* causes decarboxylation of phenolic acids and secretion of glycoamylases. The former results in "phenolic off-flavours" (Jespersen and Jakobsen, 1996). The latter results in attenuation of beer, due to the debranching enzymatic activity breaking down non-fermented dextrins (Jespersen and Jakobsen, 1996).

Pichia membranefaciens, Hansenula anomala, Debaryomyces spp., *Brettanomyces* spp., *Filobasidium* spp. and *Candida* spp. are non-*Saccharomyces* wild yeasts that contribute to spoilage (Priest and Campbell, 1987; Jespersen and Jakobsen, 1996). The majorities of wild yeasts cause turbidity and off-flavours due to phenols and haze (Priest and Campbell, 1987; Jespersen and Jakobsen, 1996).

3.1.5. Spoilage Microorganisms Associated with Sorghum Processing

MOs associated with South African sorghum beer were characterized. LAB yield was 88% of the microbial population, *Bacillus* spp. was 8.4%, *Micrococcus* spp. was 2.9% and gram negative bacteria was 0.7% (Pattison *et al*., 1998). The high population number of LAB detected may be due to the presence of LAB involved in natural lactic acid fermentation associated with sorghum beer brewing. However, only a percentage of LAB is responsible for spoilage (Pattison *et al*., 1998). For example- acetic acid produced by heterofermentative LAB contribute to spoilage (Pattison *et al*., 1998). In addition to bacteria, sorghum beer is dominated by yeasts. *Candida tropicalis, Candida kefyr,*

Kloeckera apiculata, Hansenula anomala, Torulaspors delbrueckii, Schizosaccharomyces pombe and *Kluyveromyces africanus* are examples of yeast isolates found in fermented products from sorghum (Jespersen, 2003). The microflora of sorghum beer varies dependent on geographical area of production and processing methods (Jespersen, 2003).

3.1.6. Detection of Beer Spoilage Microorganisms

The most commonly used method to detect beer spoilage MOs is culture media. This method allows the selection of the vast range of MOs present in beer (Sakamoto and Konings, 2003).

3.1.6.1. Detection of gram positive bacteria

Lactobacilli and pediococci can be detected by the use of numerous media such as de Man, Rogosa and Sharpe (MRS) agar; Raka-Ray medium; VLB S7-S and Universal Beer Agar (UBA) (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). These media are usually supplemented with cycloheximide to inhibit the growth of yeasts (Sakamoto and Konings, 2003).

UBA is suitable for detection as beer is a component of the medium. This provides the survival of the spoilage MOs and prevents the growth of non-spoilage bacterial species associated with beer (Jespersen and Jakobsen, 1996).

3.1.6.2. Detection of gram negative bacteria

Pectinatus and *Megasphaera* can be detected using MRS broth, UBA and peptone yeast extract supplemented with fructose (PYF) (Jespersen and Jakobsen, 1996; Sakamoto and Konings, 2003). Furthermore, selective medium for *Megasphaera* and *Pectinatus* (SMMP) is used for the detection of *Megasphaera cerevisiae* and *Pectinatus* spp. (Jespersen and Jakobsen, 1996). SMMP is composed of beer, nutrients, lactate, cycloheximide, crystal violet and sodium fusidate. Cycloheximide prevents yeast survival and sodium fusidate inhibits the growth of gram positive bacteria (Jespersen and Jakobsen, 1996).

Zymomonas spp. can be detected using solid or liquid media. Malt extract, yeast extract, glucose, peptone (MYGP) agar is a solid medium with 20ppm cycloheximide, 3% ethanol (v/v) and lactic acid is added to obtain a pH of 4.0 (Jespersen and Jakobsen, 1996). The liquid medium consists of glucose, fructose and yeast extract with added cycloheximide at a pH of 4.0 (Jespersen and Jakobsen, 1996).

Acetobacter spp. is detected by growth on medium composed of 0.5% (w/v) yeast extract, 1.5% (v/v) ethanol and 2.5% (w/v) agar (Jespersen and Jakobsen, 1996).

3.1.6.3. Detection of wild yeasts

Wild yeasts are difficult to detect on a single selective medium as these are biochemically and physiologically similar to culture yeasts (van der Aa Kuhle and Jespersen, 1998). Thus, a vast range of media are available for wild yeast detection.

Examples of media include:- lysine medium; cadaverin lysine ethylamine nitrate (CLEN) medium; Schwarz differential medium (SDM); Lin's wild yeast medium (LWYM); xylose mannitol adonitol cellobiose sorbitol (XMACS) medium; yeast extract, peptone, dextrose (YPD) agar and Wallernstein Laboratory Nutrient (WLN) medium (Jespersen and Jakobsen, 1996; van der Aa Kuhle and Jespersen, 1998).

Lysine medium is used for the detection of non-*Saccharomyces* wild yeasts, as they utilize lysine as a nitrogen source. *Saccharomyces* yeasts cannot grow on lysine medium (Jespersen and Jakobsen, 1996). CLEN medium is a modification of lysine medium which enables faster growth of wild yeasts. CLEN consists of numerous nitrogen sources (Jespersen and Jakobsen, 1996). SDM is composed of MYGP and a fuchsin-sulphite mixture (0.3-0.35% w/v). Fuchsin-sulphite is a selective mixture for *S. cerevisiae.* However, SDM also supports the growth of culture yeasts (Jespersen and Jakobsen, 1996). LWYM is a combination of SDM and crystal violet. LWYM is sufficient for detecting both *Saccharomyces* and non-*Saccharomyces* wild yeasts (Jespersen and Jakobsen, 1996). XMACS medium consists of a large number of carbon sources. Brewing yeasts is unable to utilize the carbon hence it is useful for the detection of *Saccharomyces* and non-*Saccharomyces* wild yeasts (Jespersen and Jakobsen, 1996). YPD is supplemented with 550ppm $CuSO_4$. $CuSO_4$ in the medium is reduced by wild yeasts. This allows for the sufficient detection of *Saccharomyces* and non-*Saccharomyces* wild yeasts. In addition, $CuSO_4$ allows for the inhibition for culture yeasts (Jespersen and Jakobsen, 1996). WLN agar is commonly used for wild yeasts present in ales. In addition, lager yeasts can be detected using WLN agar (Jespersen and Jakobsen, 1996). WLN medium is supplemented with bromocresol green, which is reduced by *Saccharomyces*

and non-*Saccharomyces* species of lager yeasts (Jespersen and Jakobsen, 1996). Reduction produces light green or blue colonies. Ales yeasts produce dark green colonies, as the bromocresol green is not reduced (Jespersen and Jakobsen, 1996).

It has been shown by van der Aa Kuhle and Jespersen (1998), that 80% of wild yeasts was detected by media containing $CuSO_4$, followed, by 46-56% detection using WLN agar (van der Aa Kuhle and Jespersen, 1998).

(3.2) Methodology

3.2.1. Experimental Design

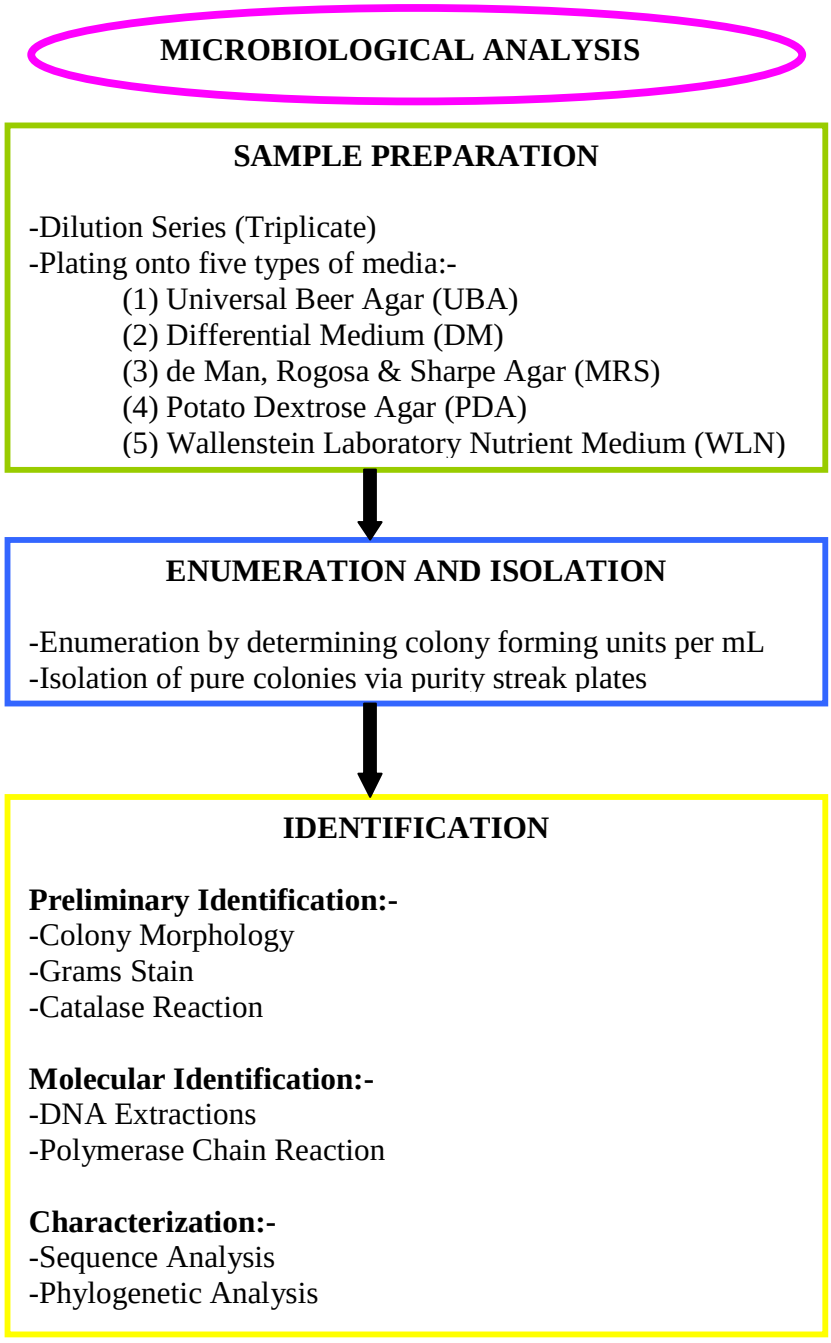


Fig 3.1 Schematic Diagram representing an outline of the Experimental Design

3.2.2. Experimental Approach

3.2.2.1. Sample Collection for Microbial Analysis

During sorghum processing (hydrolysis and fermentation) samples were collected at the post-mashing and post-fermentation stages. The samples were used for the detection of microbial contamination associated with sorghum processing at the Wits microbrewery plant. Samples were collected in re-capable, dark plastic bottles, refrigerated at 4°C and analyzed within 24h. Post-fermentation samples were collected when fermentation was complete i.e. when maximum ethanol was produced.

3.2.2.2. Sample Preparation for Microbial Analysis

From the initial liquid samples (post-mashing and post-fermentation samples), 1mL was aliquot into 9mL of sterile buffered peptone water (BPW-20g/L). Tenfold serial dilutions were performed in triplicate. The diluted suspension was used for plating, via the standard spread plate technique on to the following media-: (1) UBA (55g/L UBA agar, 750mL dH_2O, 250mL beer); (2) DM (5g/L yeast extract, 15mL absolute ethanol, 25g/L nutrient agar); (3) MRS (62g/L MRS agar); (4) PDA (30g/L PDA agar); (5) WLN (80g/L WLN medium). One hundred microlitres (100uL) of the suspension was surface-plated onto the above mentioned media. Incubation conditions are summarized in Table 3.1.

Table 3.1. Incubation conditions of the various growth media used for sample analysis

Growth Media	Temperature (°C)	Time (hours)	Atmospheric Conditions
UBA	37	24	Aerobic
DM	30	48-72	Aerobic
MRS	30	48-72	Aerobic
PDA	30	48	Aerobic
WLN	37	24	Aerobic

3.2.2.3. Enumeration and Isolation of Microbial Populations

For enumeration, plates showing between 30-300 colonies were selected and counted. Colony forming units per mL (CFU/mL), standard deviations and statistical analysis (using the t-test) for each type of media was determined between triplicate plate counts. CFU/mL was determined according to the following equation:-

$$\text{Colony Forming Units/mL} = \frac{\text{Number of Colonies X Dilution Factor}}{\text{Volume}}$$

Results were expressed as Log CFU/mL.

For isolation, morphologically different colonies were picked from the various media and inoculated into liquid broth culture. An inoculation needle was used to pick the colonies. “Putative” bacterial colonies from UBA, DM and MRS were inoculated into 10mL of Tryptone Soy Broth (TSB-30g/L TSB powder). “Putative” yeast colonies from UBA, PDA and WLN were inoculated into 10mL of Yeast Peptone Dextrose (YPD-10g/L yeast

extract, 20g/L peptone, 20g/L glucose) broth. Incubation conditions for isolation are summarized in Table 3.2.

Overnight broth cultures were purified via two successive streak plates onto the respective media from which they were initially isolated. Pure cultures were maintained on the agar plates at 4°C. In addition, pure colonies were preserved in 50% glycerol at -70°C.

Table 3.2. Incubation conditions of the liquid broth culture used for the isolation of pure colonies from each type of media

Growth Media	Liquid Broth Culture	Temperature (°C)	Time (hours)	Rotary Shaker (rpm)
UBA	TSB, YPD	37	24	170
DM	TSB	30	24	170
MRS	TSB	30	24	170
PDA	YPD	30	24	170
WLN	YPD	37	24	170

3.2.2.4. Identification Strategy of Microbial Isolates

(a) Preliminary Identification

Preliminary identification was based on:- (i) media specificity, (ii) microscopic examination by the Grams reaction and (iii) the catalase reaction.

(i) Media Specificity

Specific media used was mentioned in **3.2.2.2** and summarized in **Table 3.1.** Pure cultures grown on the media were examined for colony morphology. UBA is a selective medium used to detect several MOs associated with post-mashing (wort/sugar

hydrolyzate) and post-fermentation (beer) products of cereal grain processing as beer is one of the components (Jespersen and Jakobsen, 1996). DM was used to detect gram negative bacteria (Jespersen and Jakobsen, 1996). MRS was used for the detection of LAB (Jespersen and Jakobsen, 1996; Pattison et al., 1998). PDA was used to detect yeasts and wild yeast contaminates (Pattison et al., 1998). WLN was used specifically for the detection of wild yeast species (Jespersen and Jakobsen, 1996).

(ii) Microscopic Examination

The Gram reactions are based on a differential staining method that separates gram-positive bacteria from gram-negative bacteria (Gerhardt *et al*., 1981). The Gram stain was prepared as previously described by Gerhardt *et al*. (1981).

A pure colony was transferred onto a microscope slide and evenly smeared. The smear was heat-fixed by passing the slide through a flame. Following heat-fixation, the smear was stained (Gerhardt *et al*., 1981). For the staining procedure the smear was flooded with the following solutions:-

- Crystal Violet (5g crystal violet/L) for 1minute
- Iodine (10g iodine/L, 20g potassium iodide/L) for 1minute
- Decolourizer (210mL acetone, 90mL alcohol) was added drop wise until no colour was observed.
- Safranin (1g safranin/L) for 1minute

Between each stain the slide was rinsed with tap water and blotted dry (Gerhardt *et al*., 1981). Prepared slides were viewed under 100X objective (oil-immersion) with the use of a compound microscope (DIALUX).

(iii) The Catalase Reaction

To detect for a catalase reaction, a droplet of hydrogen peroxide (3% w/v) was added to a colony that was smeared onto a microscope slide. The presence or absence of gas bubbles was monitored (Harrigan and McCance, 1966).

(b) Molecular Identification

Molecular identification was based on:- (i) DNA extractions and (ii) Polymerase Chain Reaction (PCR) amplification.

DNA extractions and PCR cycles of "putative" bacterial isolates were performed as described by Lindsay *et al*. (2007).

(i) DNA Extraction

A single colony from pure plate cultures was inoculated, using a sterile inoculation needle, into a 1.5mL eppendorf tube containing 40uL of PCR water (See Appendix A) and 20uL chloroform. The suspension was boiled for 20 minutes and then placed on ice for 20 minutes. The DNA was then extracted by centrifugation (Eppendorf-Microcentrifuge) at 12,000 rpm for 5 minutes. Following centrifugation, the pellet was discarded and the supernatant was used as template DNA for the PCR reaction (Lindsay *et al*., 2007).

(ii) PCR Amplification

For the "putative" bacterial isolates the 16S region of rDNA was amplified with the following primers:-

- U1392R (5`-ACGGGCGGTGTGTRC-3`)
- Bac27R (5`-AGAGTTTGATGATCMTGGCTCAG-3`)

PCR amplification was carried out in a Thermal Cycler (BIORAD). The PCR reaction was performed in a final volume of 50uL using 25uL of 2X Master Mix (0.05 units/uL *Taq* DNA Polymerase in Reaction Buffer, 0.4mM dNTPs, 4mM MgCl2) with 1uL of each primer (10uM), 2.5uL of template DNA (supernatant) and 20.5uL of nuclease-free water to final volume (Lindsay *et al*., 2007). For the PCR reaction *Pseudomonas aeruginosa* was used as a positive control and water was added to the reaction mixture instead of DNA for the negative control. The positive control isolate was from the Food Microbiology Lab, School of Molecular and Cell Biology at Wits University. PCR cycles were programmed as presented in Fig 3.2 (Lindsay *et al*., 2007).

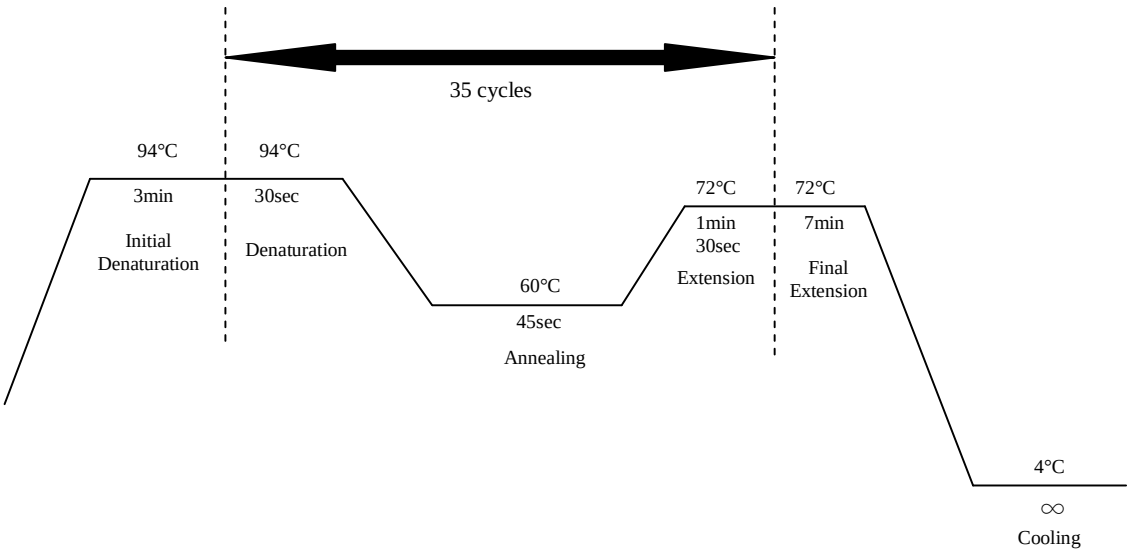


Fig 3.2 PCR Cycles of 16S rDNA Amplification

PCR products were analyzed by 1% (w/v) Agarose Gel Electrophoresis in 1X Tris-borate EDTA (TBE-BIORAD Buffer Concentrate) buffer, pre-stained with 10ug/mL of ethidium bromide. Five microlitres (5uL) of the amplified products were pre-mixed with 2uL of 6X loading dye (Fermentas) and loaded into the wells of the gel. The size of the amplified products was determined by a standard 1kb DNA ladder (GeneRuler-Fermentas). The gel was electrophorezed at 75V for 1 hour 30 minutes and viewed under a UV light equipped with a Gel Doc System.

DNA extractions and PCR reactions of "putative" yeast isolates were performed by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa. The 5.8S rDNA region was amplified using ITS 1 and ITS 4 primers (See Appendix C).

(c) Characterization

(i) Automated Sequencing

PCR products were sequenced by Inqaba Biotechnical Industries. Positive 16S rDNA amplicons were sequenced using the Bac27F primer. Positive 5.8S rDNA amplicons were sequenced using the ITS 4 primer. The sequence output files were edited using Finch TV v1.4 Software. The edited sequences were regarded as query sequences and submitted for analysis using the Blast Local Alignment Search Tool (BLAST) Software Programme (Altschul *et al*., 1990). Query sequences with ≥98% similarity were identified to species level.

(ii) Phylogenetic Analysis

Multiple sequence alignments (MSA) of the 16S and 5.8S rDNA regions of bacteria and yeast respectively were carried out using DNAMAN (Lynnon BioSoft version 4.0).

For the alignments, the partial nucleotide sequences of the isolates obtained in this study was aligned to partial nucleotide sequences of species that were previously identified with mashing and fermentation of sorghum processing (Jespersen and Jakobsen, 1996; van der Aa Kuhle and Jespersen, 1998; Naumova *et al*., 2003; Sakamoto and Konings, 2003; Basilio *et al*., 2008). In addition, sequences identified for each isolate, according to BLAST were used for the alignment to compare relatedness. Partial sequences of previously identified species was obtained from GenBank. The comparative species and their corresponding GenBank accession numbers are shown in Tables 3.3 and 3.4. From the MSA, phylogenetic trees were constructed using DNAMAN. Bootstrap value of 100 was selected and branch lengths were computed by the DNAMAN Software Programme.

Table 3.3 A list of bacterial species and GenBank accession numbers

BACTERIAL SPECIES	GENBANK ACCESSION NUMBERS	REFERENCES
Lactobacillus brevis	EF 412995	Sakamoto and Konings, 2003; Jespersen and Jakobsen, 1996
Lactobacillus buchneri	AF 429586	Sakamoto and Konings, 2003
Lactobacillus casei	AB 507114	Sakamoto and Konings, 2003
Lactobacillus plantarum	AF 429550	Sakamoto and Konings, 2003
Lactobacillus curvatus	AF 318167	Sakamoto and Konings, 2003
Pediococcus damnosus	AF 429536	Sakamoto and Konings, 2003; Jespersen and Jakobsen, 1996
Pediococcus acidilactici	DQ 104396	Sakamoto and Konings, 2003
Pediococcus dextrinicus	FM 877685	Sakamoto and Konings, 2003
Pediococcus inopinatus	NR 025388	Sakamoto and Konings, 2003; Jespersen and Jakobsen, 1996
Lactococcus lactis	FJ 749561	BLAST Analysis
Enterococcus faecalis	FJ 804073	BLAST Analysis
Enterococcus faecalis	FJ 804073	BLAST Analysis
Lactococcus lactis	FJ 749561	BLAST Analysis
Lactobacillus casei	GQ 131245	BLAST Analysis
Lactococcus lactis	FJ 749561	BLAST Analysis
Lactococcus garvieae	AB 244455	BLAST Analysis
Lactococcus lactis	FJ 749561	BLAST Analysis
Lactococcus garvieae	AB 244455	BLAST Analysis
Lactobacillus casei	GQ 131245	BLAST Analysis

Table 3.4. A list of yeast species and GenBank accession numbers

YEAST SPECIES	GENBANK ACCESSION NUMBERS	REFERENCES
Candida tropicalis	FJ 011533	Basilio *et al*., 2008
Candida biodinii	EF 197945	Van der Aa Kuhle and Jespersen, 1998
Pichia membranaefaciens	EJ 231463	Van der Aa Kuhle and Jespersen, 1998
Dekkera bruxellensis	EU 014766	Basilio *et al*., 2008
Hanseniaspora guilliermondii	FJ 231466	Van der Aa Kuhle and Jespersen, 1998
Zygosaccharomyces fermentati	AY 046206	Van der Aa Kuhle and Jespersen, 1998
Saccharomyces bayanus	EU 145763	Naumova *et al*., 2003
Saccharomyces cariocanus	AY 046147	Naumova *et al*., 2003
Saccharomyces mikatae	AB 040996	Naumova *et al*., 2003
Saccharomyces pastorianus	AB 279757	BLAST Analysis
Saccharomyces cerevisiae NCL117	AM 262831	BLAST Analysis
Saccharomyces cerevisiae NCL117	AM 262831	BLAST Analysis
Saccharomyces kudriavzevii	FJ 873454	BLAST Analysis
Saccharomyces cerevisiae strain T8	FJ 838776	BLAST Analysis
Issatchenkia orientalis	AB 467299	BLAST Analysis
Saccharomyces pastorianus	AB 279757	BLAST Analysis
Saccahromyces paradoxus	FJ 713072	BLAST Analysis
Issatchenkia orientalis	AB 467299	BLAST Analysis
Candida inconspicua	EU 315758	BLAST Analysis

(3.3) Results and Discussion

For the purpose of this investigation enumeration and identification of MOs is essential to the maintenance of the Wits microbrewery plant. Furthermore, microbial contaminants associated with sorghum processing for bioethanol production is a key component for controlling the effect of MOs in the manufacturing process.

In this study, microbiological examination was at the post-mashing (hydrolysis with the use of commercial exogenous enzymes) and post-fermentation stages. Microbial analysis referred to as SP1 and SP2 is post-fermentation analysis of samples using *S. cerevisiae* and *I. orientalis*, respectively, for fermentation.

3.3.1. Microbial Counts

At the post-mashing stage of SP1 and SP2, plate counts were insignificant as <30 colonies were observed on the various types of detection media. This could possibly be due to the high mashing temperatures being unfavourable for the growth of MOs. Pattison *et al*. (1998) suggests that boiling at temperatures between 80°C to 96°C is capable of killing vegetative MOs. Furthermore, tannins of sorghum grains are known to exhibit antimicrobial activity (Suleiman *et al*., 2007). In the current study, the condensed tannins of the bitter sorghum variety were not removed prior to hydrolysis and therefore may account for the absence of MOs at the post-mashing stage.

At the post-fermentation stage of SP1 and SP2, there are numerous MOs associated with the process, indicated by the mean aerobic plate counts (Log CFU/mL) (Fig 3.3). Plate counts are statistically significant ($p<0.05$).

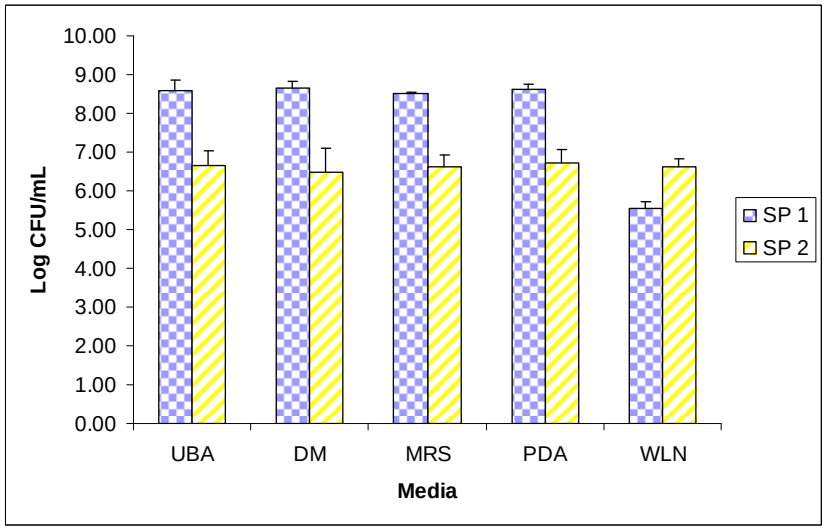


Fig 3.3. Log CFU/mL of bacterial and yeast cells on various types of media at the post-fermentation stage. Error bars indicate standard deviation between triplicate plate counts. ($p<0.05$).

Mean aerobic plate counts of 8.85, 8.67, 8.51, 8.63 and 5.56 Log CFU/mL were obtained for UBA, DM, MRS, PDA and WLN, respectively, for SP1 (Fig 3.3). Bacterial counts (UBA, DM, MRS) and yeast counts of PDA were similar and significantly higher than the yeast counts of WLN (Fig 3.3).

For SP2, mean aerobic plate counts of 6.67, 6.49, 6.62, 6.71 and 6.61 Log CFU/mL were obtained from UBA, DM, MRS, PDA and WLN, respectively (Fig 3.3). Plate counts are almost identical for all types of media (Fig 3.3).

Several microbial populations detected by the plate counts suggest that a range of MOs are able to survive and proliferate under optimum fermentation conditions. The microbial counts decreased significantly ($p<0.05$) from ~9 to ~7 Log CFU/mL from SP1 to SP2 (Fig 3.3). Post-fermentation analysis was carried out at day 5 for SP1 and at day 12 for SP2, as these were the time points at which maximum ethanol was achieved and

considered to be the end of fermentation. Numerous factors such as:- decreasing nutrients and oxygen availability; increasing alcohol and carbon dioxide and an acidic environment could favour the decline in the microbial counts as the duration of fermentation increases. WLN yeast counts of SP1 shows the lowest count compared to the other microbial populations (Fig 3.3). However, WLN yeast counts of SP2 increased with an increase in fermentation time (Fig 3.3) WLN (SP2) microbial populations are able to reach similar levels of UBA, DM, MRS and PDA populations (Fig 3.3) at day 12 of fermentation. This indicates that certain yeast populations can adapt and increase in number under restricted conditions. By comparison, microbial counts obtained in this study are similar to results observed by Pattison *et al*. (1998) and Mugula *et al*. (2003a). In these studies, plate counts of bacteria ranged from ~6 to ~9 Log CFU/mL and yeast counts ranged from ~5 to ~7 Log CFU/mL (Pattison *et al*., 1998; Mugula *et al*., 2003a).

3.3.2. Identification of Microbial Populations

3.3.2.1. Preliminary Identification

The microbial populations isolated at the post-fermentation stage of SP1 and SP2 were preliminarily identified by the analysis of colony morphology on specific media, Grams reaction and cell shape and catalase reaction. Preliminary identification results are summarized in Tables 3.5 and 3.6. From the selective media, 20 MOs were isolated and their characteristics are represented in Tables 3.5 and 3.6. Examination of MOs from the various media of the post-fermentation of SP1 and SP2 (Figs 3.4 and 3.5) showed gram positive cocci and rods (Figs 3.6) and catalase negative reactions of isolates from UBA, DM and MRS (Figs 3.4a-c and 3.5b-c). Isolates from PDA and WLN of SP1 and SP2

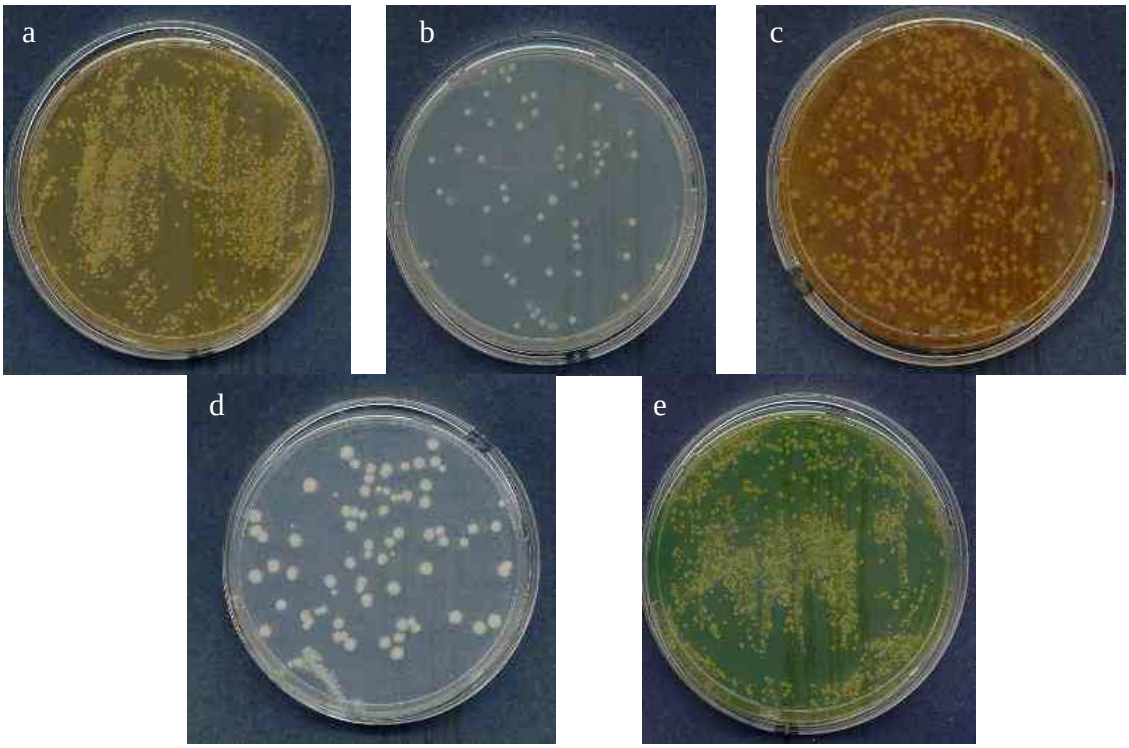
(Figs 3.4d-e and 3.5d-e) and UBA of SP2 (Fig 3.5a) stained gram positive and are catalase positive. Grams reaction of yeast isolates showed budding yeasts, cylindrical cells and spherical cells (Figs 3.6). Colony morphology on media showed bacterial colonies is medium in size, white in colour, circular in shape with smooth margins (Figs 3.4 and 3.5). Yeast colonies are large in size, cream or white in colour, circular in shape with smooth or undulate margins (Figs 3.4 and 3.5).

Table 3.5 Preliminary Characteristics of SP1 isolates based on colony morphology, grams reaction, cell shape and catalase reaction.

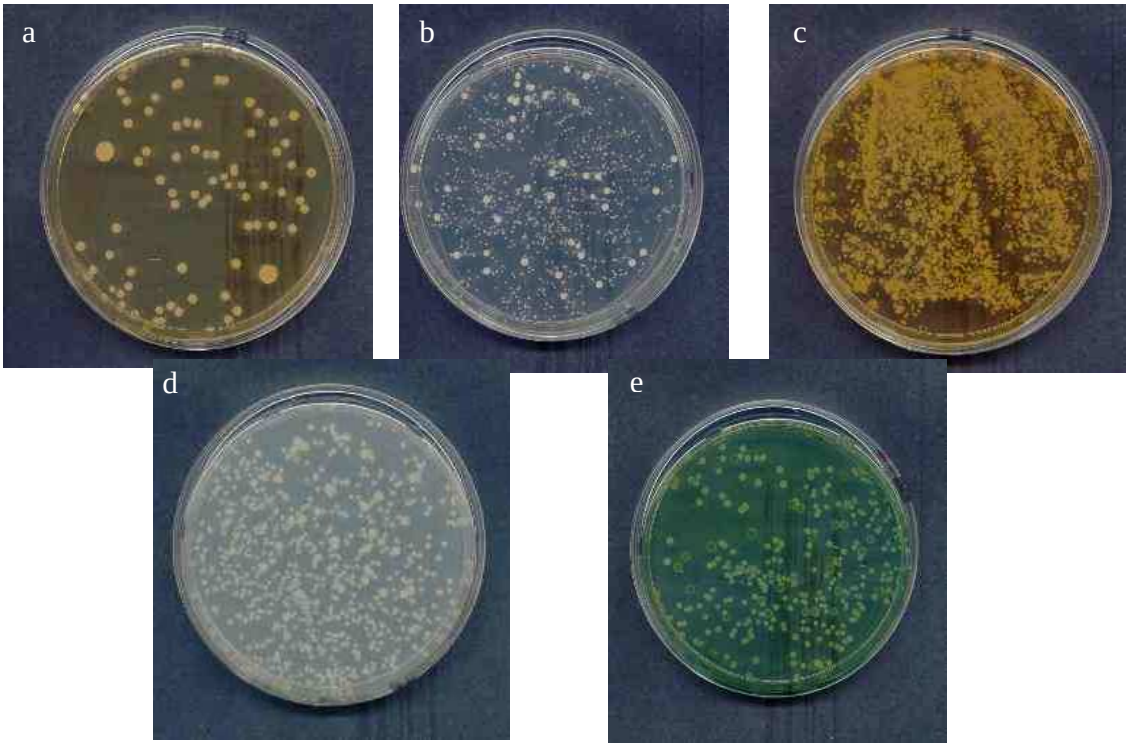
Growth Media	Colony Morphology	Grams Reaction and Cell Shape	Catalase Reaction
UBA	(1) circular, white (2) circular, white	(1) positive, cocci (2) positive, cocci	(1) negative (2) negative
DM	(1) circular, white (2) circular, white	(1) positive, cocci (2) positive, cocci	(1) negative (2) negative
MRS	(1) circular, white (2) circular, white	(1) positive, rods (2) positive, cocci	(1) negative (2) negative
PDA	(1) circular, white (2) circular, white	(1) positive, budding yeasts (2) positive, budding yeasts	(1) negative (2) negative
WLN	(1) circular, white (2) circular, cream	(1) positive, budding yeasts (2) positive, budding yeasts	(1) negative (2) negative

Table 3.6. Preliminary Characteristics of SP2 isolates based on colony morphology, grams reaction, cell shape and catalase reaction

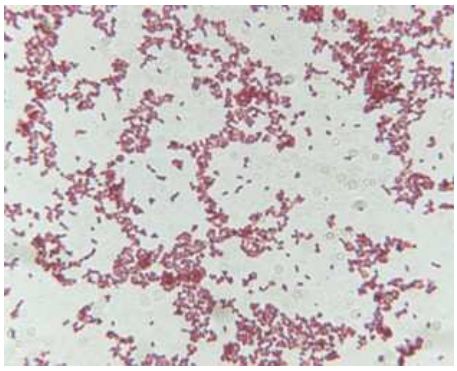
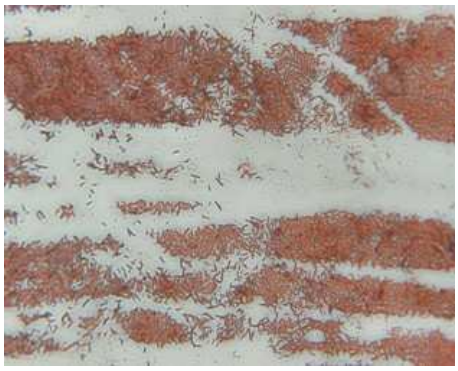
Growth Media	Colony Morphology	Grams Reaction and Cell Shape	Catalase Reaction
UBA	(1) circular, white (2) undulate, white	(1) positive, budding yeasts (2) positive, cylindrical cells	(1) positive (2) positive
DM	(1) circular, white (2) circular, white	(1) positive, cocci (2) positive, cocci	(1) negative (2) negative
MRS	(1) circular, white (2) circular, white	(1) positive, cocci (2) positive, rods	(1) negative (2) negative
PDA	(1) circular, white (2) circular, white	(1) positive, budding yeasts (2) positive, budding yeasts	(1) positive (2) positive
WLN	(1) undulate, white (2) circular, yellow	(1) positive, cylindrical cells (2) positive, spherical cells	(1) positive (2) positive

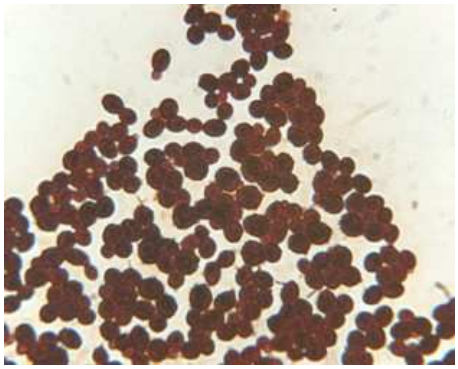
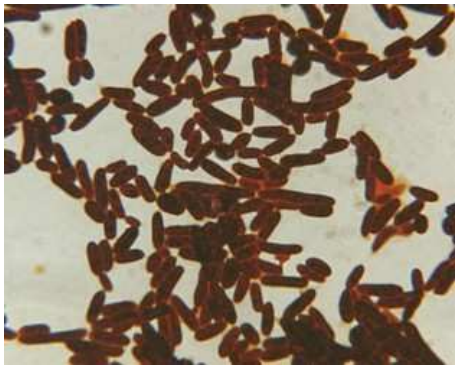


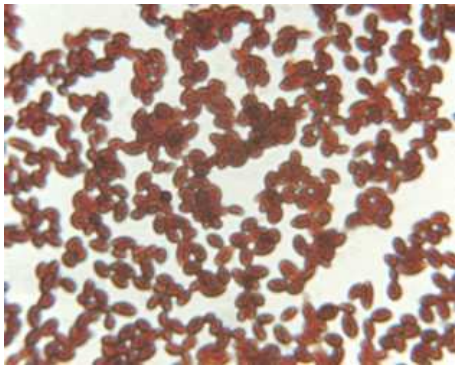
Figs 3.4a-e. Various types of media showing microbial populations associated with sorghum processing at the post-fermentation stage of SP1 (a) UBA, (b) DM, (c) MRS, (d) PDA, (e) WLN. (a-c) bacterial colonies, as UBA, DM and MRS are media specific for visualization of bacterial growth. (d-e) yeast and wild yeast colonies, as PDA and WLN are media specific for the visualization of yeast growth.



Figs 3.5a-e. Various types of media showing microbial populations associated with sorghum processing at the post-fermentation stage of SP2 (a) UBA, (b) DM, (c) MRS, (d) PDA, (e) WLN. (b-c) bacterial colonies, as DM and MRS are media specific for visualization of bacterial growth. (a,d,e): yeast and wild yeast colonies, as UBA, PDA and WLN are media specific for the visualization of yeast growth.

(a)  (b) 

(c)  (d) 

(e) 

Figs 3.6a-e. Gram stains of isolates. (a) gram positive, cocci. (b) gram positive, rods. (c) budding yeasts. (d) cylindrical cells (e) spherical cells

UBA is effective for the growth of bacteria (SP1) and yeasts (SP2). DM showed the isolation of possible LAB which was unexpected, as DM is selective for gram negative bacteria (Jespersen and Jakobsen, 1996). Gram negative spoilage bacteria are unable to tolerate alcohol concentrations ≥5% (w/v) and low concentrations of gram negative bacteria are detected in sorghum fermentations (Jespersen and Jakobsen, 1996; Pattison *et al*., 1998). The medium could be supplemented with crystal violet or sodium fusidate to prevent the growth of gram positive bacteria (Jespersen and Jakobsen, 1996). MRS showed positive LAB growth which is in agreement with previous reports involving detection media for the isolation of LAB (Jespersen and Jakobsen, 1996; Pattison *et al*., 1998). PDA and WLN media were able to detect *Saccharomyces* and non-*Saccharomyces* yeasts.

The preliminary identification of the 20 isolates, based on morphological and biochemical characteristics suggest that the isolates could be grouped as LAB, *Saccharomyces* and non-*Saccharomyces* wild yeasts. Preliminary grouping of the isolates from the current study is consistent with isolates previously identified from sorghum fermented products (Pattison *et al*., 1998; van der Aa Kuhle and Jespersen, 1998; Mugula *et al*., 2003a).

3.3.2.2. Molecular Identification

To ascertain UBA (SP1), DM (SP1, SP2) and MRS (SP1, SP2) isolates were in fact bacterial isolates, PCR amplification was performed. The 16S rDNA region of the isolates was amplified using U1392R and Bac27F primers. Template DNA was extracted from plate colonies (Figs 3.4 and 3.5).

The 16S region of all isolates shows identical DNA fragment size of 1500bp (Fig 3.7).

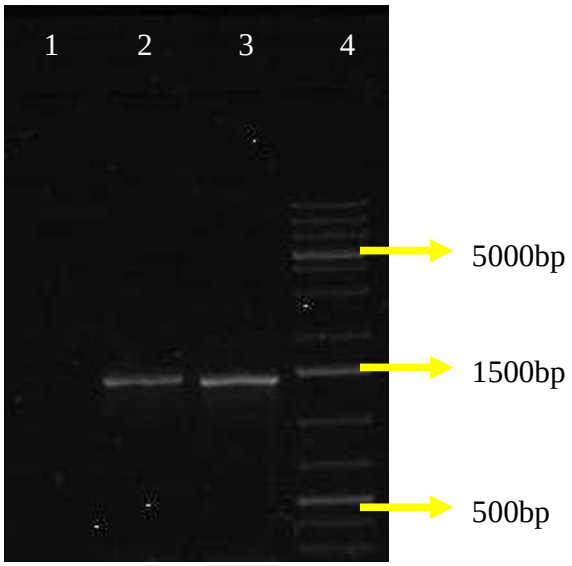


Fig 3.7 Agarose Gel of 1500bp DNA fragments amplified by 16S rDNA primers of UBA, DM and MRS isolates. Lane 1: Negative Control. Lane 2: Positive Control. Lane 3: MRS isolate. Lane 4: Molecular Weight Marker (1kb DNA Ladder-Fermentas).

The PCR technique proved successful for the detection of positive bacterial isolates as the positive control (Lane 2-Fig 3.7) shows positive amplification of a 1500bp product and thus eliminates the possibility of non-specific amplification.

The remaining isolates from UBA (SP2), PDA (SP1, SP2) and WLN (SP1, SP2) were verified as yeast isolates by the amplification of the 5.8S rDNA region using ITS 1 and ITS 4 primers (results not shown).

3.3.2.3. Characterization of Microbial Isolates

(a) Automated Sequencing

PCR products were sequenced. The query sequences were compared to sequences available in the National Centre for Biotechnology Information (NCBI) GenBank database by BLAST analysis. Identification of the isolates was documented if the query sequence showed ≥98% identity.

A total of 20 isolates were recovered from the use of the Wits microbrewery plant for bioethanol production. A summary of the bacterial and yeast sequence identification, based on partial sequence analysis of the 16S and ITS regions, respectively is represented in Table 3.7.

Table 3.7. Sequence identification of bacterial and yeast isolates from different types of media of SP1 and SP2.

Isolates*	% Similarity	DNA Sequence Identification	Length of Query Sequence
1	99%	*Lactococcus lactis* subsp. lactis strain	716
2	99%	*Enterococcus faecalis* strain CTC 328	823
3	100%	*Enterococcus faecalis* strain CTC 328	604
4	99%	*Lactococcus lactis*	832
5	100%	*Lactobacillus casei*	702
6	100%	*Lactococcus lactis*	801
7	98%	*Saccharomyces pastorianus*	168
8	99%	*Saccharomyces cerevisiae* NCL117	196
9	99%	*Saccharomyces cerevisiae* NCL117	198
10	98%	*Saccharomyes kudriazevii*	180
11	100%	*Saccharomyces cerevisiae* strain T8	347
12	100%	*Issatchenkia orientalis*	360
13	100%	*Lactococcus garvieae*	249
14	100%	*Lactococcus lactis*	108
15	99%	*Lactococcus garvieae*	640
16	99%	*Lactobacillus casei*	814
17	99%	*Saccharomyces pastorianus*	184
18	100%	*Saccharomyces paradoxus*	130
19	98%	*Issatchenkia orientalis*	590
20	98%	*Candida inconspicua*	178

*Isolates 1-10 were obtained at the post-fermentation stage of SP1. Isolates 11-20 were obtained at the post-fermentation of SP2.

The sequences revealed that the isolates from UBA (SP1), DM and MRS (isolates 1-6 and 13-16) were lactic acid bacteria (LAB) (Table.3.7). The occurrence of *L.casei* (isolates 5 and 16) coincides with LAB previously identified from traditional sorghum beer (Pattison *et al*., 1998). The current study is the first to report the isolation of *L.lactis* and *L.garvieae* using sorghum as a raw material for bioethanol production. The presence of *E. faecalis* is unusual and possibly is a contaminant of the water (Santo Domingo *et al*., 2003), rather than a product of the fermentation process. *L. lactis* and *E.faecalis* was previously isolated from milk and in addition were used for the fermentation of milk (Muguerza *et al*., 2006; Narvhus *et al*., 1998). In addition, *Lactococcus* spp. were identified as inhabitants of beer, contributing to spoilage (Suzuki *et al*., 2008).

Sequence analysis of isolates from UBA (SP2), PDA and WLN (isolates 7-12 and 17-20) showed predominately *Saccharomyes* yeasts (Table.3.7). The non-*Saccharomyces* yeasts isolated were *C. inconspicua* (isolate 20) and *I.orientalis* (isolates 12 and 19). However, *I.orientalis* was likely isolated as it was added as a starter culture for bioethanol processing. Wild yeasts belonging to the genus *Candida* account for >30% of the contamination in distilleries using cane sugar for bioethanol production (Basilio *et al*., 2008). Basilio *et al.* (2008) suggests that *Candida* spp. could contribute to a decline in the yield of bioethanol. Yeast species identified in the present study correspond to the *sensu stricto* strains of species commonly associated with African sorghum beer (Naumova *et al*., 2003), with the exception of *Saccharomyces cariocanus* and *Saccharomyces mikatae.* The consistent isolation of *S.cerevisiae* strains was expected as 55-90% of *S.cerevisiae* is inhabitants of sorghum beer (Naumova *et al,* 2003) and thus would naturally be found to be the dominant microbial community at the post-fermentation stage from sorghum

processing for bioethanol production. *S.kudriazevii* is rarely isolated from fermentation processes involving the use of sorghum raw materials, as this species showed a high incidence of contamination during the production of wine (Gonzalez *et al*., 2008). It has been reported that low fermentation temperatures support the growth of *S.kudriavezii* (Gonzalez *et al*., 2008), in this study the fermentation carried out at 19◦C was sufficient for the survival of this particular MO.

A variety of yeast species were isolated and identified in comparison to bacterial species. This could possibly be due to the LAB contributing to a decline in pH (Mugula *et al*., 2003b). The resulting acidic environment is favourable for proliferation of yeasts. In addition, *S.cerevisiae* which is introduced during produces growth stimulants such as amino acids, vitamins and pyruvate, which in turn serves as favourable factors for the growth of other yeast species (Jespersen, 2003). The microbial community present at the post-fermentation stage of sorghum processing in relation to bioethanol production is multifactorial as contamination could be due to unsterilised use of the grain, addition of water, low pH and temperature, enzymatic activity which contributes to the availability of sugary substrates that can easily be assimilated by MOs and equipment used during the production process such as pipes and vessels could be contributing factors. Furthermore, productivity of bioethanol can be hindered by biological instability (Basilio *et al*., 2008) as there will be substrate antagonism between wild yeast species and yeast species used essentially for fermentation.

(b) Phylogenetic Analysis

To evaluate the evolutionary relatedness of the bacterial and yeast isolates retrieved in this study, phylogenetic trees were constructed using the 16S and 5.8S rDNA regions.

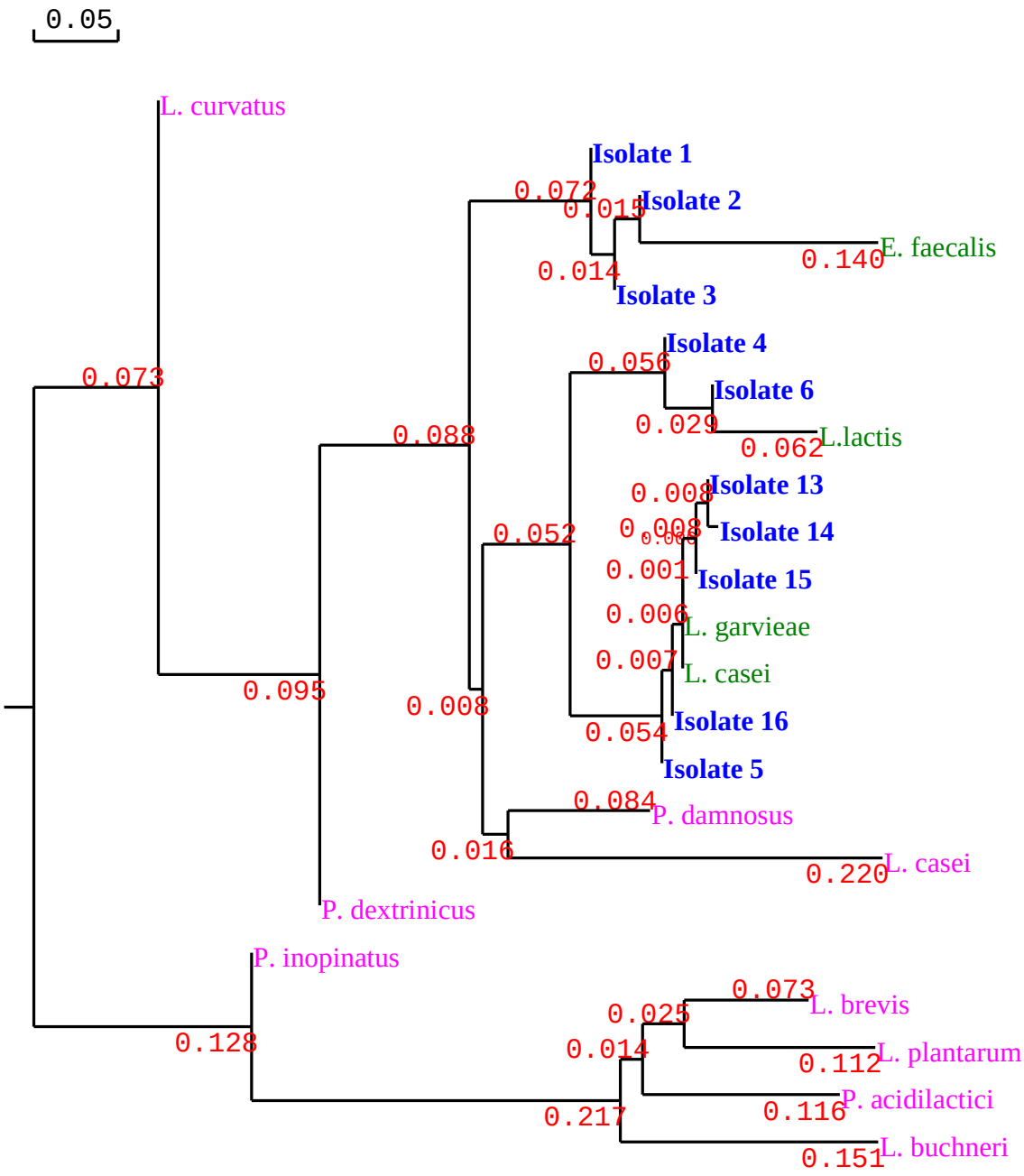


Fig 3.8. Phylogeny of the 16S region. The tree is represented as a rooted tree with branch lengths computed by using DNAMAN. The scale bar is a statistical value as a result of a bootstrap value of 100. Isolates from this study is indicated in BLUE. BLAST identified sequences are indicated in GREEN. Sequences obtained from GenBank are indicated in PURPLE.

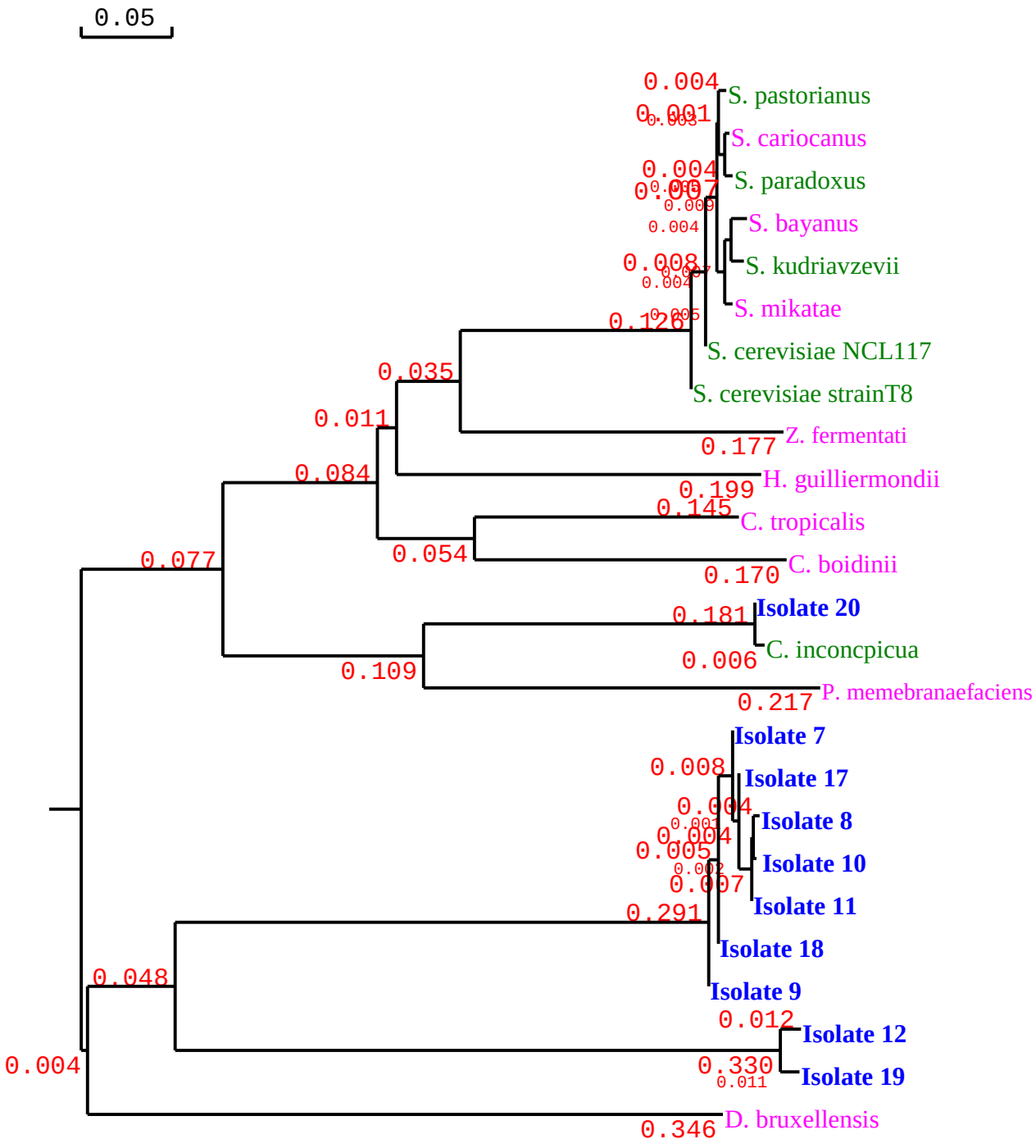


Fig 3.9. Phylogeny of the 5.8S region. The tree is represented as a rooted tree with branch lengths computed using DNAMAN. The scale bar is a statistical value as a result of a bootstrap of 100. Labeling is as indicated in Figure 3.8.

Tree construction was based on MSA between the 20 isolates and sequences obtained from GenBank (NCBI database).

The resulting phylogenetic tree (Fig 3.8) shows bacterial isolates from the current investigation are distantly related to LAB previously identified in fermented products. Isolates 1, 2 and 3 show clustering and is rooted with *E. faecalis* (Fig 3.8). However, isolate 1, identified as *L. lactis* did not cluster with isolates 4, 6 and 14, which were also identified as *L. lactis* (Table 3.7). Thus isolate 1 is a different strain as a result of the development of different genes through evolution. Isolate 14 is clustered with isolates 13 and 15 and this strain of *L. lactis* is closely related to *L. garvieae* (Fig 3.8). *L. casei* isolates (5 and 16) are distantly related to previously identified *L. casei* (Fig 3.8).

The 5.8S phylogenetic tree shows yeast isolates from this investigation are rooted but distantly related to yeasts previously identified (Fig 3.9). As expected, isolates 7, 8, 9, 10, 11, 17 and 18 are clustered (Fig 3.9) as these were identified as *Saccharomyces* species (Table 3.7). Isolates 12 and 19 is rooted to *D. bruxellensis* (Fig 3.9). Branch lengths of the *I. orientalis* isolates and *D. bruxellensis* are almost equal meaning that these species evolved at similar rates through evolution with minor changes in the gene sequence.

(3.4) Conclusions

In conclusion results of the present study showed:-

- High bacterial and yeast counts at the post-fermentation stage of SP1 and SP2 was detected and thus there are numerous MOs that are able to proliferate within the fermentation conditions used for bioethanol production.
- Yeast counts detected by WLN of SP2 was higher than the counts obtained for SP1, therefore yeast contamination is favoured over bacteria as fermentation time increases.
- Preliminary identification based on characteristics such as colony morphology, Grams reaction and cell shape and catalase reaction allowed isolates recovered from the various detection media were grouped into LAB, *Saccharomyces* yeasts and non-*Saccharomyces* wild yeasts.
- Molecular identification resulted in positive 16S amplification products of 1500bp from UBA (SP1), DM (SP1, SP2) and MRS (SP1, SP2) isolates.
- Sequence identification revealed the presence of *L. lactis, L. garvieae, L. casei. E. faecalis, C. inconspicua, I. orientalis, S. cerevisiae* strain T8, *S. cerevisiae* NCL117, *S. pastorianus, S. paradoxus, S. kudriazveii.*
- Phylogenetic analysis of 16S and 5.8S rDNA regions showed that the isolates from the current study were closely related to each other but distantly related to previously identified species associated with fermentation.

(3.5) References

Altschul, S.F., Gish, W., Miller, W., Meyer, E.W. and Lipman, D.J. (1990). Basic Local Alignment Search Tool. *Journal of Molecular Biology.* **215(3):** 403-410.

Basilio, A.C.M., de Araujo, P.R.L., de Morais, J.O.F., da Silvio Filho, E.A., de Morais, M.A. and Simoes, D.A. (2008). Detection and Identification of Wild Yeast Contaminants of the Industrial Fuel Ethanol Fermentation Process. *Current Microbiology*. **56:** 322-326.

Coton, M., Laplace, J.M., Guichard, H. and Coton, E. (2008). Factors Affecting *Zymomonas mobilis* subsp. *francensis* Growth and Acetaldehyde Production. *Journal of the Institute of Brewing.* **114(2):** 114-121.

Gerhardt, P., Murray, R.G.E., Costilow, R.N., Nester, E.W., Wood, N.A., Krieg, N.R. and Phillips, G.B. Manual Methods for General Microbiology. American Society for Microbiology. Washington, DC, 1981, pp: 21-33.

Gonzalez, S.S., Barrio, E. and Querol, A. (2008). Molecular Characterization of New Natural Hybrids of *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* in Brewing. *Applied Environmental Microbiology*. **74(8):** 2314-2320.

Gram, L., Ravn, L., Rasch, M., Bruln, J.B., Chistensen, A.B. and Givskov, M. (2002). Food Spoilage-interactions between food spoilage bacteria. *International Journal of Food Microbiology*. **78:** 79-97.

Harrigan, W.F. and McCance, M.E. Laboratory Methods in Microbiology. Academic Press, London and New York, 1966, pp: 65.

Jespersen, L. and Jakobsen, M. (1996). Specific spoilage organisms in breweries and laboratory media for their detection. *International Journal of Food Microbiology.* **33:** 139-155.

Jespersen, L. (2003). Occurrence and taxonomic characteristics of strains of Saccharomyces cerevisiae predominant in African indigenous fermented foods and beverages. *FEMS Yeast Research*. **3:** 191-200.

Lindsay, D., Ntoampe, M. and Gray, V.M. (2007). Biodegradation of sodium benzoate by a Gram negative consortium in a laboratory-scale fluidized bed reactor. *Bioresource Technology*. (doi:10.1016/j. biortech. 2007.09.020).

Manzano, M., Guisto, C., Bartolomeoli, I., Buiathi, S. and Comi, G. (2005). Microbiological Aanlyses of Dry and Slurry Yeasts for Brewing. *Journal of the Institute of Brewing.* **111(2):** 203-208.

Muguerza, B., Ramos, M., Sanchez, E., Manso, M.A., Miguel, M., Aleixandre, A., Delgado, M.A. and Recio, I. (2006). Antihypertensive activity of milk fermented by Enterococcus faecalis strains isolated from raw milk. *International Dairy Journal*. **16:** 61-69.

Mugula, J.K., Nnko, S.A.M., Narvhus, J.A. and Sorhaug, T. (2003a). Microbiological and Fermentation Characteristics of *togwa,* a Tanzanian fermented food. *International Journal of Food Microbiology*. **80:** 187-199.

Mugula, J.K., Narvhus, J.A. and Sorhaug, T. (2003b). Use of starter cultures of lactic acid bacteria and yeasts in the preparation of *togwa,* a Tanzanian fermented food. *International Journal of Food Microbiology*. **83:** 307-318.

Narvhus, J.A., Osteraas, K., Mutukumira, T. and Abrahamsen, R.K. (1998). Production of fermented milk using a malty- compound- producing strain of *Lactococcus lactis* subsp. lactis biovar. *diacetylactis*, isolated from Zimbabwean naturally fermented milk. *International Journal of Food Microbiology*. **41:** 73-80.

Naumova, E.S., Korshunova, I.V., Jespersen, L. and Naumov, G.I. (2003). Molecular genetic identification of *Saccharomyces* sensu stricto strains from African sorghum beer. *FEMS Yeast Research*. **3:** 177-184.

Pattison, T., Geornaras, I., and von Holy, A. (1998). Microbial populations associated with commercially produced South African sorghum beer as determined by conventional and petrifilm plating. *International Journal of Food Microbiology.* **43:** 115-122.

Priest, F.G. and Campbell, I. Brewing Microbiology. Elsevier Applied Science Publishers LTD, 1987, pp: 122-202.

Sakamoto, K. and Konings, W.N. (2003). Beer Spoilage Bacteria and Hop Resistance. *International Journal of Food Microbiology*. **33:** 139-155.

Santo Domingo, J.W., Siefrieg, S.C. and Haugland, R.A. (2003). Real-time PCR method to detect *Enterococcus faecalis* in water. *Biotechnology Letters*. **25:** 261-265.

Suleiman, A.M.E., Issa, F.M. and Elkhalifa, E.A. (2007). Quantitative Determination of Tannin Content in some Sorghum Cultivars and Evaluation of its Antimicrobial Activity. *Research Journal of Microbiology*. **2(3):** 284-288.

Suzuki, K., Asano, S., Iijima, K. and Kitamoto, K. (2008). Sake and Beer Spoilage Lactic Acid Bacteria-A Review. *Journal of the Institute of Brewing.* **114(3):** 209-223.

Van der Aa Kuhle, A. and Jespersen, L. (1998). Detection and Identification of wild yeasts in lager breweries. *International Journal of Food Microbiology*. **43:** 205-213.

CHAPTER 4:
CONCLUDING REMARKS AND FUTURE RECOMMENDATIONS

(4.1) Concluding Remarks

The current research was undertaken with the aim of investigating the level of bioethanol produced from bitter sorghum grains hydrolyzed by commercial exogenous enzymes and subsequent fermentation and to evaluate the microbiological quality of sorghum processing by the use of the Wits microbrewery plant. The evidence supports the idea that bioethanol production can be achieved from un-malted bitter sorghum grains via the use of Cerezyme Sorghum and Fungamyl 800L. Dual-enzymatic hydrolysis assisted to enhance the bioethanol level from 3% (v/v) to 7% (v/v). The approach used in this study proved successful for bioethanol production and the microbrewery plant is efficient. However, since the concentration of ethanol is economically unsuitable for distillation, as described in previous literature, there is a requirement to further optimize hydrolysis and fermentation for the commercialization for this particular enzyme combination with the Red Swazi variety of sorghum grains. Furthermore, the high amount of maltose might restrict the utilization by the yeast. Another factor that was considered for this research was microbiological contamination, as this indicates hygienic operational processes and maintenance of the plant. In addition, it is important with respective to bioethanol as certain microbial inhabitants can contribute to a decrease in the concentration of ethanol. In this study, the microbial community was identified based on morphological and biochemical characteristics as well as molecular methods. Characteristics showed microorganisms such as LAB, *Saccharomyces* and non-*Saccharomyces* yeasts were

recovered from post-fermentation analysis. The presence of the contaminants possibly competes for nutrients with the yeasts added for the fermentation and in turn effects the yield of bioethanol. There was inconsistency in the identification of the microorganisms between SP1 (fermentation using *S. cerevisiae*) and SP2 (fermentation using *I. orientalis*) and thus the sources of contamination could be the sugar hydrolyzate, or the connecting pipes used for transferring the liquids, or the fermentation vessel. Microbiological analysis represents knowledge on the microorganism inhabitants present during sorghum processing for bioethanol production with the use of the Wits microbrewery plant.

(4.2) Future Recommendations

- Enzyme binding assays to detect if activity is affected by the presence of sorghum grain tannins.
- Optimization of mashing and fermentation conditions to increase the bioethanol yield.
- Implementing strategies to reduce the number of MOs present during processing.
- Measuring the consumption of fermentable sugars to determine the fermentation effectivity.

APPENDIX A:
PUBLICATIONS, PRESENTATIONS and OTHER ACTIVIES DURING THE INVESTIGATION

Publications

1. Deenanath, E.D., Iyuke, S.E. and Lindsay, D. (2009). Enzymatic Production of Bioethanol from Bitter Sorghum. Submitted for publication to the *Journal of the Institute of Brewing*, awaiting review.
2. Deenanath, E.D., Iyuke, S.E. and Lindsay, D. (2009). Identification of microbial populations associated with sorghum fermentation. Submitted for publication to the *MBAA Technical Quarterly*, awaiting review.

Presentations

3. Deenanath, E.D., Iyuke, S.E. and Lindsay, D. (2009). Enzymatic Production of Bioethanol from Bitter Sorghum, South African Chemical Engineering Conference (SACEC), Conference for Sustainable Engineering Development, Stellenbosch, South Africa, September 2009. Manuscript published in conference proceedings.

Other Activities

4. Member of the Institute of Brewing and Distilling and a Brewing Diploma Candidate. Attended lectures for examinations of three modules, Wits University, South Africa, February 2008-June 2008.
5. Awarded a pass in the IBD exam for Module 1: Raw Materials and Wort by the Institute of Brewing and Distilling, UK, June 2008.

6. Participated in the Intervarsity Microbrewery Competition sponsored by South African Breweries, Institute of Brewing and Distilling and Food BevSETA, August 2008 (Pietermaritzburg) and August 2009 (Pretoria).

   August 2008: Wits University was awarded second position in the Ale Category.

   August 2009: Wits University was awarded second position in the Ale and Specialty Categories.

Patents

7. Deenanath, E.D., Iyuke, S.E., Lindsay, D. (2009). The Isolation of a Wild Yeast Species for its Application in Bioethanol Production, Subject of Provisional Patent. Date of Application: 2009-10-14. Submission Number: 2009/07104.

APPENDIX B:
RECIPES FOR MEDIA, STAINS and SOLUTIONS

The methods used for the preparation of the media, stains and solutions used in this research are given below.

MEDIA

All media used was prepared from a premixed powder. Media was purchased from Merck Laboratories in its ready-made form.

(1) Universal Beer Agar (UBA)

A selective medium for the isolation of spoilage MOs associated with beer.

UBA Powder……. 55 g

Distilled Water…….. 750 mL

Beer……..... 250 mL

Dissolve UBA in 750mL distilled water by simultaneously heating and stirring. Once pellets have dissolved, add beer without degassing and continue stirring. Sterilize by autoclaving at 121°C for 20 minutes.

(2) Differential Medium (DM)

Used for the isolation of gram negative bacteria.

Yeast Extract Powder……... 5 g

Absolute Ethanol…….. 15 mL

Nutrient Agar……... 25 g

Distilled Water……. 985 mL

Dissolve all ingredients and sterilize by autoclaving at 121°C for 20 minutes.

(3) de Man, Rogosa & Sharpe (MRS) Agar

A specific medium used for the isolation of Lactic Acid Bacteria

MRS Agar…… 62 g

Distilled Water…… 1000 mL

Dissolved ready-made agar in its powder form with distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(4) Potato Dextrose Agar (PDA)

Used for the isolation of yeasts.

PDA Powder…… 39 g

Distilled Water……. 1000 mL

Dissolve premixed agar in 1L distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(5) Wallenstein Laboratory Nutrient (WLN) Medium

Used for the isolation of wild yeasts.

WLN Powder……... 80 g

Distilled Water……. 1000 mL

Dissolve premixed media in 1L distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(6) Malt Extract Agar (MEA)

Used for the isolation of a pure *S. cerevisiae* isolate from a packet of dry brewers yeast and to detect pitching rate for fermentation.

MEA Powder…… 50 g

Distilled Water……. 1000 mL

Dissolve premixed powder in 1L distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(7) Buffered Peptone Water (BPW)

Used as a diluent.

BPW Powder…… 20 g

Distilled Water……. 1000 mL

Dissolve powder in 1L distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(8) Tryptone Soy Broth (TSB)

Used as a liquid broth culture for the growth of bacterial isolates to obtain pure cultures.

TSB Powder……. 30 g

Distilled Water……. 10000 mL

Dissolve powder in 1L distilled water and sterilize by autoclaving at 121°C for 20 minutes.

(9) Yeast Peptone Dextrose (YPD) Broth

Used as a liquid broth culture for the growth of yeasts to obtain pure cultures.

Yeast Extract Powder……... 10 g

Peptone……. 20 g

Glucose……. 20 g

Distilled Water……. 1000 mL

Dissolve yeast extract powder and peptone in 950mL of distilled water and sterilize by autoclaving at 121°C for 20 minutes.

Dissolve glucose in 50mL of distilled water and do not autoclave.

Once broth is cool, filter sterilize 50mL of glucose solution into the broth culture to obtain a final volume of 1L.

STAINS

Gram stain solutions were prepared to detect for a grams reaction. Stock solutions of the stains were stored at room temperature.

(1) Crystal Violet

Crystal Violet Powder…….. 5 g

Distilled Water……. 1000 mL

(2) Iodine

Iodine	10 g
Potassium Iodide	20 g
Distilled Water	1000 mL

(3) Safranin (1% Solution)

Safranin…… 1 g

Distilled Water……. 1000 mL

(4) Decolourizer (30% Solution)

Acetone..... 210 mL

Distilled Water……. 90 mL

SOLUTIONS

Other solutions used during the research

(1) PCR Water

Wash schott bottles (X2) with 2M HCL and then rinse with distilled water. Spray bottles with 70% ethanol and rinse once again with distilled water. Autoclave bottles at 121°C for 20 minutes. Once bottles are dry and free of condensation droplets, add distilled water in one schott bottle and autoclave at 121°C for 20 minutes. Post-sterilization, filter sterilize the water in to the second schott bottle. Aliquot as required.

(2) 2M Hydrochloric Acid

Used to eliminate protein contamination from Schott bottles.

32% HCL……. 64 mL

Distilled Water……. 36 mL

(3) Bleaching Reagent

Used to detect tannins of sorghum grains. Stock solution can be stored at room temperature for a duration of 1 month.

Sodium Hydroxide……... 5 g

Commercial Bleach…….. 100 mL

(4) Tris-borate EDTA (TBE) Buffer

Used in agarose gel electrophoresis for the mobility of DNA.

10X TBE Buffer Concentrate from BIORAD.

Buffer Concentrate……... 100mL

Distilled Water……. 900mL

Final concentration of 1X TBE solution: 89mM Tris, 89mM boric acid, 2mM EDTA, pH 8.4

(5) Hydrogen Peroxide

Used to detect for a catalase reaction. Working concentration is 3% (w/v).

30% (w/v) Stock Hydrogen Peroxide…….. 1 mL

Distilled Water……. 9 mL

(6) Sodium Chloride

Used to re-suspend cultured yeast. Concentration was 0.85% (w/v).

Sodium Chloride Pellets…….. 0.85 g

Distilled Water……. 100 mL

Dissolve and filter sterilize.

(7) Glycerol

Used to preserve pure bacterial and yeast isolates recovered during the research. Working concentration was 50%.

Glycerol……. 25 mL

Distilled Water…….. 25 mL

Dissolve and autoclave at 121°C for 20 minutes.

APPENDIX C:
PCR CYLCES of YEAST AMPLIFICATION

Yeast DNA extraction and PCR amplification was performed by Inqaba Biotechnical Industries, Pty (Ltd), Pretoria, South Africa. The primers used were ITS 1 and ITS 4. Primer sequences were as follows:-

- ITS 1 (5`- TCCGTAGGTGAACCTGCGG-3`)
- ITS 4 (5`-TCCTCCGCTTATTGATATGC-3`)

The PCR cycles were programmed as represented in Figure 1.

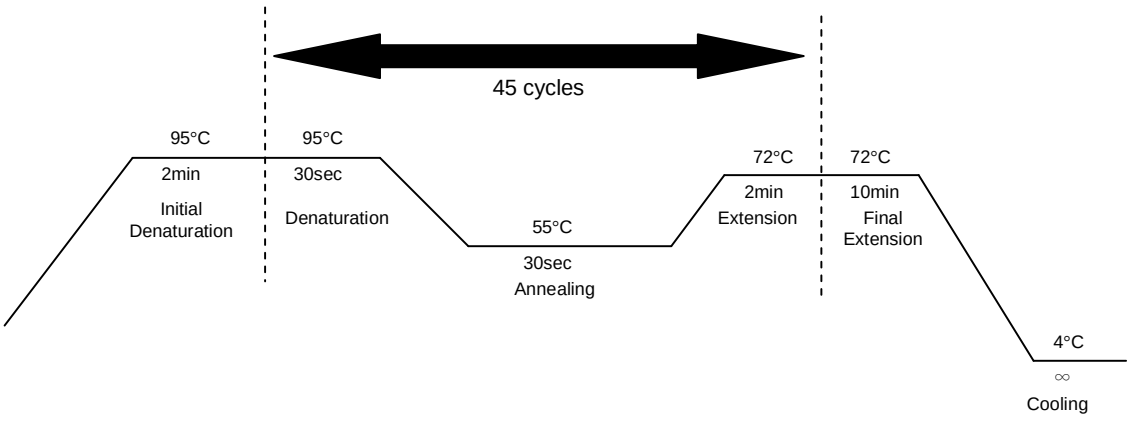


Fig 1. PCR cycles for the amplification of the 5.8S rDNA region of yeast isolates from UBA (SP1), PDA (SP1, SP2) and WLN (SP1, SP2).

APPENDIX D:
RAW DATA

Values presented in this section were used for the construction of graphs that are depicted in Chapter 2 and Chapter 3.

Tables 1a-c. Raw data used to construct Fig 2.12.

Table 1a. Raw data for SP1

Fermentation Time in Days	Alcohol Gay-Lussac % (v/v)
1	0%
2	0%
3	4%
4	6%
5	7%

Table 1b. Raw data for the control

Fermentation Time in Days	Alcohol Gay-Lussac % (v/v)
1	0%
2	0%
3	0%
4	2%
5	3%

Table 1c. Raw data for SP2

Fermentation Time in Days	Alcohol Gay-Lussac % (v/v)
1	0%
2	0%
3	0%
4	0%
5	0%
6	1%
7	2%
8	4%
9	4%
10	5%
11	6%
12	7%

Tables 2a-b. Data used to construct Fig 3.3.

Table 2a. Raw data from plate counts of SP1 and SP2

Media	Dilution Factor	Replicates			Mean	CFU/ml
SP1		**1**	**2**	**3**		
UBA						
	1*E-05	295	201	210	235	2.35E+08
	1*E-06	74	58	61	61	6.10E+08
DM						
	1*E-05	324	396	351	357	3.57E+08
	1*E-06	60	52	73	62	6.20E+08
MRS						
	1*E-05	301	291	290	294	2.94E+08
	1*E-06	38	35	33	35	3.50E+08
PDA						
	1*E-05	320	360	348	343	3.43E+08
	1*E-06	50	54	49	51	5.10E+08
WLN						
	1*E-02	270	284	271	275	2.75E+08
	1*E-03	36	54	51	47	4.70E+08
SP2						
UBA						
	1*E-03	275	240	256	257	2.57E+06
	1*E-04	99	79	78	85	8.50E+06
DM						
	1*E-03	129	98	110	112	1.12E+06
	1*E-04	75	83	80	83	8.30E+06
MRS						
	1*E-03	270	248	241	253	2.53E+06
	1*E-04	61	75	70	69	6.90E+06
PDA						
	1*E-03	289	291	280	287	2.87E+06
	1*E-04	92	93	90	92	9.20E+06
WLN						
	1*E-03	276	292	270	279	2.79E+06
	1*E-04	54	62	59	58	5.80E+06

Table 2b. Data used to construct Fig 3.3.

Log CFU/ml	Average Log CFU/ml	p-values	SD
SP1			
8.37	8.58	0.020809	0.296985
8.79			
8.55	8.67	0.006904	0.169706
8.79			
8.47	8.505	7.14E-05	0.049497
8.54			
8.54	8.625	0.001425	0.120208
8.71			
5.44	5.555	0.000333	0.162635
5.67			
SP2			
6.41	6.67	0.000975	0.367696
6.93			
6.05	6.485	0.100854	0.615183
6.92			
6.4	6.62	0.004456	0.311127
6.84			
6.46	6.71	0.000167	0.353553
6.96			
6.45	6.605	0.00062	0.219203
6.76			

Tables 3a-b. Raw data used to detect the pitching rate of *S. cerevisiae* and *I.orientalis*

Table 3a. Raw data of *S. cerevisiae*

Dilution factor	Number of Colonies		Colony Forming Units/mL	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
10^{-1}	TNTC	TNTC	-	-
10^{-2}	TNTC	TNTC	-	-
10^{-3}	TNTC	TNTC	-	-
10^{-4}	TNTC	TNTC	-	-
10^{-5}	670	572	$6.70X10^{8}$	$5.72X10^{8}$
10^{-6}	93	71	$9.30X10^{8}$	$7.10X10^{8}$

Table 3b. Raw data of *I.orientalis*

Dilution factor	Number of Colonies		Colony Forming Units/mL	
	Replicate 1	Replicate 2	Replicate 1	Replicate 2
10^{-1}	TNTC	TNTC	-	-
10^{-2}	TNTC	TNTC	-	-
10^{-3}	150	175	$1.50X10^{6}$	$1.75X10^{6}$
10^{-4}	99	85	$9.90X10^{6}$	$8.50X10^{6}$
10^{-5}	<30	<30	-	-
10^{-6}	<30	<30	-	-